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THE ALGERNON B. REESE

Proctor Medal Award

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-seventh Meeting

San Francisco, California

June 23-26, 1958

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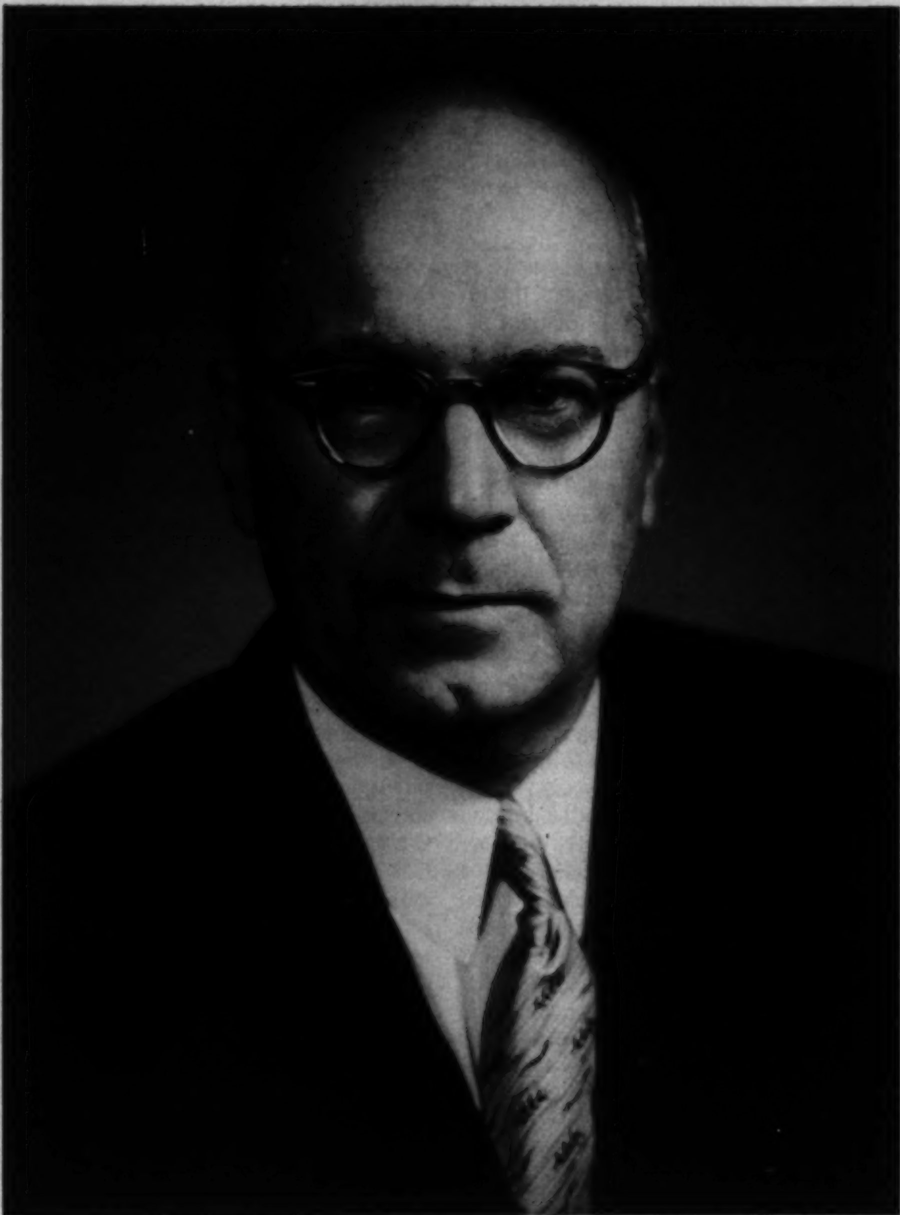
THE PROCTOR MEDAL

Funds for the establishment of the Research Medal of the Association for Research in Ophthalmology were donated in 1947 by Mrs. Francis I. Proctor of Santa Fe, New Mexico, as a memorial to her late husband. Dr. Proctor, a Boston ophthalmologist, became intensely interested in the experimental side of ophthalmology after his retirement, and participated in numerous studies on the etiology and treatment of trachoma. The purpose of the medal is to stimulate research and to honor investigators who have made notable contributions in the basic fields of ophthalmology. The medal is to be awarded without regard to the nationality or professional status of the recipient.



Fabian Bachrach

ALGERNON BEVERLY REESE, M.D.

ALGERNON BEVERLY REESE

An appreciation

The Association for Research in Ophthalmology has designated Algernon Reese as the 1958 Laureate for the Proctor Medal. No choice could be happier, no award more richly merited. My heartiest and most heartfelt congratulations to both the association and to Dr. Reese.

To be asked to write a biographic sketch of Algernon Reese while he is in the full flush of lusty manhood and at the height of his career, is in itself a unique privilege. Too often these sketches are melancholy obituaries, the recital of past accomplishments which have become meaningless, a dirge in honor of a departed loved one. Now it is a joyous opportunity to pay tribute to a respected, an honored, and a dearly loved companion. Therefore allow me to depart from the usual hackneyed form of these brief biographies, and write an appreciation of the man as I know him.

Algernon Reese was born and raised down in North Carolina, a state which is known as the "Valley of Humility" between two adjoining "Mountains of Conceit"—Virginia and South Carolina. Maybe this is the reason why, despite all his later accomplishments, he has remained the unassuming, honest, and lovable character he is today. His mother was a North Carolinian, a member of a family some of whom had attained great wealth as manufacturers, an achievement in which Mrs. Reese's particular branch unfortunately did not participate. His father was born a Virginian. He was graduated in pharmacy and moved to Charlotte, North Carolina, where he had a drugstore. His descendants describe him as "a lovable gentleman, with wonderful instincts and a good sense of humor, but not a good provider." This may be paraphrased as saying he was a delightful, courteous, generous, and improvident Virginia gentleman—of which there are so many! The result was that the Reese family lived under a rigid economy, Mrs. Reese, the North Carolinian, doling out what funds there were, with the education of her children an ever present and almost insoluble problem. This family history is important when we try to understand Algeron as the great ophthalmologist he is today.

The paternal grandfather was also a Virginian, a stalwart Confederate and a most extraordinary character. It was through his influence that Algernon became a doctor. The name of this grandfather was Beverly Patrick Reese. He died in 1918 at the age of 94 years. At the time of his death he was the oldest living graduate of Princeton University, which he had attended in the halcyon antebellum days of Virginia. It is notable, however, that when his alma mater tried to collect biographic data on their venerable patriarch, that he wrote back "You damn Yankees, mind your own business!" And that was that! He was a country doctor in the Valley of Virginia, one of that stalwart and now unfortunately vanished tribe who found their greatness and immortality in the hearts of their patients whom they tirelessly and unselfishly served, in wind and weather, day in and day out, often without remuneration, until at long last the grim reaper gathered them to their final resting place. He was a proud and magnificently handsome man. He invariably wore a high "plug" hat and drove through the country on his rounds in a two-horse rig, chiefly because no single horse could possibly have hauled a buggy through the then frightful and mountainous roads of Augusta County. It was this grandfather that young Al Reese visited through the summers of his boyhood, making rounds with him through the countryside and mountains of Virginia, and listening to medical stories told by his grandfather. One can visualize a wide-eyed and impressionable boy, observing first-hand the problems of medicine and the ills of patients, how some were treated with pills, others with a scalpel or a splint, and, when

there was no hope, how they were consoled, encouraged, and fortified by understanding and kindness. It was from grandfather Beverly Patrick Reese that young Algernon began to learn medicine at the very fountainhead, and under his influence that he became dedicated irrevocably to a medical career.

Algernon received his early education at public schools, with a final year at a private school which he candidly admits "was terrible." He was a well co-ordinated youngster and excelled at sports, a fact his fellow ophthalmologists have learned to their sorrow when in later years they have vainly attempted to extract money from him at golf and tennis! At Davidson College, he modestly states he was a poor student. This is almost certainly an inaccurate statement, for it is noteworthy he made Phi Beta Kappa, an attainment which may possibly have been due to his excellence as a basketball player, although one is permitted to doubt this. In any event, either his scholastic record, his athletic prowess, or his personality were sufficient to engage the interest of both his wealthly maternal uncle and his ophthalmologist paternal uncle, the late Dr. Robert Grigg Reese. The former offered to sponsor his education and take him into his extensive and lucrative business on a father-son basis. The latter countered this by an offer to finance his medical education and training as an ophthalmologist on certain strict and rigorous conditions which were later specifically spelled out. The choice of careers was left to the young man. Not for a moment did he hesitate. The choice had already been made in those summers spent with his grandfather. The life of a business tycoon was politely declined, and he accepted the long years of study and training to become an ophthalmologist—the decision to enter a specialty being made before he even began the study of medicine! And all this was accepted under conditions so rigorous they would have intimidated a lesser man than Algernon B. Reese.

The letter from Dr. R. G. Reese to his brother outlining his proposition is preserved in the archives of the Reese family, and it is so remarkable that it should be publicized. In part it reads as follows:

> My dear Brother: I want you to think seriously of persuading Algernon, Jr., to study medicine. It is my ambition to make him the greatest ophthalmologist in America and I am positive I can do it if you will allow me to direct his course. He would not be able to practice or earn a cent until he is thirty years old—I will gladly lend a helping hand. . . . He must have an A.B. degree from Yale or Harvard so it would be necessary to start him at one of these colleges without delay. An academic degree from Davidson would be of no advantage to him whatever if he intends to locate in New York and that is naturally what I want him to do. . . . I thought of giving . . . this opportunity but I am afraid he is too apathetic and not mentally equal to the occasion. . . I hope you and Mame will not allow your devotion to Algernon to stand in his way for a brilliant future.

After the blind acceptance of this offer, the details were later meticulously spelled out. On only one point did Algernon depart from his uncle's program. He refused to leave Davidson and scorned both Yale and Harvard. It is notable that for this act of allegiance Davidson later awarded him the honorary degree of Doctor of Science! The program outlined for this future ophthalmologist was four years at the Harvard Medical School, six months of study abroad before beginning an internship, a two years' internship in general surgery, six months' training with Verhoeff in Boston in eye pathology before beginning a service at the New York Eye and Ear Infirmary, two years in ophthalmology as interne and resident at that institution, then to Europe to study under Hofrat Ernst Fuchs for one year before returning to New York to enter practice with his uncle, and finally to become "the greatest ophthalmologist in America"! For his medical education and this long training Dr. R. G. Reese promised to advance the necessary funds, receiving back from Algernon promissory notes at six-percent interest, payable after he began practice. And hereby hangs a tale!

Algernon Reese followed every detail of this long program. He made a distinguished record at the Harvard Medical School, studied abroad for six months, took

a two years' general surgical service at the Roosevelt Hospital in New York, was initiated in ocular pathology by Verhoeff for six months, took a two years' service at the New York Eye and Ear Infirmary, and then went to Vienna for a year's study with the great Hofrat. He then returned to New York and moved into his uncle's house on West 52nd Street. Here he began the practice of ophthalmology as an assistant to Dr. Robert Reese.

Whether the future "greatest ophthalmologist in America" would have gotten along with his dictatorial uncle is something no one will ever know. Having known Dr. Robert Reese slightly in my younger years, and having known Algernon well in my later years, I have grave doubts. But these doubts will never be resolved. Whether it was a week in practice with his erudite nephew, the Dempsey-Tunney fight in a rainstorm in Philadelphia which Dr. Robert Reese attended, a subsequent sinusitis and either a meningitis or a brain abcess, the fact remains that Dr. Robert Reese died exactly nine days after Algernon's return to America.

In his will, Dr. Robert Reese stipulated that all indebtedness due his estate from his nephew be cancelled on condition that Algernon would pay 20 percent of his net earnings to Dr. Reese's widow as long as she lived. This proposition was accepted by the then penniless Algernon. Oh foolish young man! Never was there so costly a medical education and ophthalmological training purchased in all the history of American medicine! Algernon Reese went on from success to success to become one of America's great ophthalmologists. Mrs. Robert Reese lived for 20 years to draw down her full 20 percent of his net income each year! Be it said to his eternal credit, Algernon discharged his obligations with honor and without complaint, even when the sums received by Mrs. Robert Reese reached fantastic amounts compared with the relatively small original indebtedness. He paid his debt to his uncle in full.

I have told this intimate story of Algernon's family and training because to me it gives an understanding of the dedication, the motivation, the selflessness, and the sterling honesty of the man—qualities which have been strongly reflected in his professional, scientific, and social life. To me it is a splendid story, and one which all those who know, honor, and love him should know. I trust he will find it in his heart to forgive me for thus telling his family secrets.

The death of Dr. Robert Reese marked the real turning point in Algernon's life. That his uncle had planned wisely for him cannot be gainsaid, but Algernon had thus far followed a path laid down by someone else. Now for the first time he was suddenly independent, free to choose for himself, the master of his own fate.

At that time there were several services at the New York Eye and Ear Infirmary. In one group Dr. Robert Reese was the dominant figure, and in the other first Dr. Weeks and later Dr. John Wheeler. In the normal course of events, Algernon would have gone on as a junior man on the service which gave allegiance to his uncle. Perhaps Uncle Robert had trained his nephew too well, perhaps the stimulation of the months with Verhoeff still lingered, perhaps the year of hero worship under the great Fuchs still stirred nostalgic memories, but, whatever it was, it was soon clear that there was no real happiness or contentment for Algernon Reese as a junior man on any of the services to which he was predestined. At that time the new Institute of Ophthalmology at the Presbyterian Hospital had just been completed and Dr. John Wheeler had been appointed head. To him went Algernon and applied for a job. Dr. Wheeler told him he could join the staff but made no promises. And so began a happy, a fruitful, and a productive life, first under Wheeler and later under Dr. John Dunnington, two magnificent and stimulating men, who gave to Algernon Reese full scope for the development of his many talents, and who found in him a splendid lieutenant and department head, and also

a joyous companion and associate.

In a short time Dr. Wheeler asked Algernon to organize a department of ocular pathology, and a year later made him Pathologist to the Institute and still later surgeon. At the Institute he supervised the preparation and study of all pathologic material, organized weekly clinical-pathologic conferences, and began his correlation of clinical and pathologic findings. His many reports in this field are well known to all of us. Many of them are classic and have become an accepted part of ophthalmic literature. Notable among these are his contributions to our knowledge of retinal dysplasia, of persistent hyperplastic primary vitreous, of the pathology of retrolental fibroplasia, of telangiectasis of the retina—to mention but a few of his exhaustive and excellent studies.

I cannot pass over these many solid contributions without mentioning one which illustrates Algernon Reese's sterling intellectual honesty and his willingness to admit when he is wrong. In the early days of the adrenal corticosteroid therapy, all of us were groping pretty much in the dark, amazed at, and certainly not clearly understanding what we observed. At that time, in his Proctor Lecture, Dr. Reese reported his findings in 14 babies with retrolental fibroplasia who were treated with corticotropin, and in eight similar babies who were left untreated as controls. Peculiarly enough, the process appeared to be arrested in the treated babies, while it progressed relentlessly in the untreated controls. On this observation, and some rather pretty philosophic reasoning, he built up a strong case for steroid therapy in this disease. However, further experience with corticotropin in retrolental fibroplasia gradually convinced him that he had erred in his first conclusion. The following year, where it would receive the greatest possible publicity, he frankly admitted his mistake, stated that in his later experience he had become convinced that corticotropin was postively contraindicated in the treatment of retrolental fibroplasia, and urged against its use. Now every investigator is likely to make mistakes, and the history of medicine bristles with examples of able men who have drawn erroneous conclusions from honest observations. The usual tendency is to rectify quietly the error as far as possible in subsequent communications. It takes a high moral courage and a rugged intellectual honesty to come forward voluntarily and admit publicly the error of one's ways! This courage and honesty Algernon Reese had.

In 1928 he was appointed ophthalmologist to the Memorial Center for Cancer and Allied Diseases. Here he had an unsurpassed opportunity to study the entire range of neoplastic disease. In the ensuing years he acquired a broad knowledge and perspective of the entire field of oncology. This is abundantly reflected in his excellent treatise on *Tumors of the Eye*, published in 1951, which will long remain a classic in our ophthalmic literature.

He had long been intensely interested in the question of retinoblastoma and the possible therapy of this frightful disease. Working with Dr. Hayes E. Martin who, Dr. Reese states, "spark-plugged the early studies," trials with irradiation therapy had yielded encouraging, but in many ways inconclusive and unsatisfactory results. About 1951, he began to study the possibilities of chemotherapy, especially with nitrogen mustard and its allied compounds, such as triethylene melamine (TEM). He soon became convinced that along these lines there was definite hope for a solution of the problem. With dogged determination he persevered, trying first one and then another method of administration, finally culminating in the intracarotid injection. As a result of his efforts a Department of Radiotherapy was established in the Institute of Ophthalmology. This has now become almost a national center for both the radiation and chemotherapy of retinoblastoma. To say the early results have been remarkable is an understatement. If they are confirmed by the follow-up and later observation of the pa-

tients, Algernon Reese's name will go down in medical history. I think that if one asked him what has been his greatest contribution to ophthalmology, he would reply: "I think it was probably my pig-headedness in keeping on with attempted therapy in retinoblastoma!"

In the meantime, with all these long hours and days of study, of clinical histopathologic correlation, of teaching, and of writing, he has grown steadily in stature, as an ophthalmic surgeon, as a clinician, and as a consultant. It would be manifestly absurd to say that he has fulfilled the somewhat grandiose prophesy of his Uncle Robert, and has become "the greatest ophthalmologist in America." There are too many eager competitors for this title, and I refuse to play favorites. But it is undoubtedly true that he has become one of America's great ophthalmologists, a splendid surgeon, a careful and experienced clinician, a valued consultant, honored and respected by all who know him. He takes his place among the elect.

I have spoken thus far only of his training, professional attainments, and his fundamental qualities of character. There is yet another phase which one must know in order to get a proper picture of the man as a whole. This is his delightful social side, his modesty, his simplicity, his generosity, his thoughtfulness for his friends, and for the younger men—and possibly his absentmindedness! They have endeared him to his colleagues and to his fellow ophthalmologists as are few men in our profession. But probably the nicest thing of all about him is his sense of humor—and his laughter is just as spontaneous and sincere when the joke is on him as it is when on someone else!

He has been the recipient of honors which should come to any man of his distinction—notable are an LL.D. from Duke University and the presidency of the American Academy of Ophthalmology and of other societies. He has given various name lectures both in this country and abroad. For those who are interested, all of these are duly recorded in *Who's Who*. But, thank Heaven, this is not an obituary, and there is no need to list them here. There is one great honor, however, which must be mentioned.

In 1942, the beautiful and charming Joan Leeds finally succumbed to Algernon's blandishments, and consented to become his wife, to bear his children, and to endeavor to keep him in order for the rest of his natural life. And what a splendid job she has done of it! As he has grown in stature, she has grown in grace and loveliness. Sometimes he becomes broadminded and takes her to medical meetings with him—to the great delight of all his friends. But all too often she refuses to leave their three children. Those of his friends who are privileged to visit their home, find a warm welcome, a happy and contented family, and an unending hospitality. Perhaps the secret of this happiness lies in what she writes about him—"He is a really nice guy, gallant, affable, and dependable. He works hard, but he knows how to relax, too, and he has an endearing sense of humor. His sons and I adore him." Can anything be nicer for a wife to say about her husband?

If there is some Valhalla where tired doctors go when their toil is done, and from which they can look down on their followers and descendants, then I am sure Uncle Robert will look down and be quite pleased with what he doubtless feels is his own particular handiwork. And Grandfather Beverly Patrick Reese, with a smile on his face under his plug hat, and with a glow and a smile in his heart, will look down on his favorite grandson, and be proud that the boy's dedication to medicine was not in vain and that he has kept the faith. I do not think the old gentleman will care particularly whether or not Algernon has become the greatest ophthalmologist in America, but he will rejoice that he became, and remains, a real doctor!

Alan C. Woods.

Articles published

1. Observations on protargin strong as a preoperative prophylactic. J.A.M.A., **82**:1593-1595 (May 17) 1924.
2. Recurrent tumor of brow with unusual histologic findings. Am. J. Ophth., **8**:353-354 (May) 1925.
3. Melanosis oculi: A case with microscopic findings. Am. J. Ophth., **8**:865-870 (Nov.) 1925.
4. A useful eye bandage. J.A.M.A., **85**:1305 (Oct.) 1925.
5. Abscesses of the optic nerve. Arch. Ophth., **56**:265-271 (May) 1927.
6. Acquired ectropium uveae. Am. J. Ophth., **10**:586-593 (Aug.) 1927.
7. Entropium uveae. Am. J. Ophth., **10**:818-823 (Nov.) 1927.
8. Pigment deposit in the contraction furrows of the iris. Arch. Ophth., **2**:27-29 (July) 1929.
9. Extension of glioma (retinoblastoma) into the optic nerve. N.Y.E.&E. Inf. Clin. Rep., Mar., 1930; Arch. Ophth., **5**:269-271 (Feb.) 1931.
10. Peripapillary detachment of the retina accompanying papilledema (thesis). Tr. Am. Ophth. Soc., **28**:341-351, 1930.
11. Intrascleral nerve loops. Tr. Am. Ophth. Soc., **29**:148-153, 1931; Arch. Ophth., **6**:698-703 (Nov.) 1931.
12. A useful device for an ophthalmoscope. Am. J. Ophth., **15**:346-347 (Apr.) 1932.
13. Exophthalmos. Its pathology and ocular manifestations. N.Y. State J. Med., **33**:73-77 (Jan.) 1933.
14. Errors in diagnosis. Am. J. Ophth., **16**:151 (Feb.) 1933.
15. Iridic processes. Arch. Ophth., **10**:713-714 (Nov.) 1933.
16. Pigmentation of the optic nerve. Tr. Am. Ophth. Soc., **30**:338-343, 1932; Arch. Ophth., **9**:560-570 (Apr.) 1933.
17. Exophthalmos in leukemia. (With Loren Guy.) Am. J. Ophth., **16**:718-720 (Aug.) 1933.
18. The pathology of chronic simple glaucoma. N.Y. State J. Med., **33**:1428-1431 (Dec.) 1933.
19. The ciliary processes: Their relation to intraocular surgery. A.M.A., Tr. Sect. Ophth. 1933, pp. 262-277; Am. J. Ophth., **17**:422-428 (May) 1934.
20. Gonioscopic findings after Elliot operation. (With M. U. Troncoso.) Tr. Ophth. Soc. U. Kingdom, **53**:366-391, 1933; Am. J. Ophth., **18**:103-119 (Feb.) 1935.
21. Neurofibromatosis with ocular changes and involvement of the thoracic spine. (With M. M. Copeland and L. F. Craver.) Arch. Surg., **29**:108-112 (July) 1934.
22. The occurrence of ciliary processes on the iris. Tr. Am. Ophth. Soc., **32**:127-130, 1934; Am. J. Ophth., **18**:6-9 (Jan.) 1935.
23. Symposium: Exophthalmos. Its eye complications; Its causes from primary lesions in the orbit; Its Surgery. Tr. Am. Acad. Ophth., **39**:65-80, 1934; Arch. Ophth., **14**:41-52 (July) 1935.
24. Dacryadenitis in hyperthyroidism. Arch. Ophth., **13**:855-857 (May) 1935.
25. Massive retinal fibrosis in children. Am. J. Ophth., **19**:576-582 (July) 1936.
26. The operative treatment of concomitant squint. J. M. Soc. New Jersey, **33**:159-162 (Mar.) 1936.
27. Treatment of retinal gliomas by the fractionated or divided dose principle of roentgen radiation: Preliminary report. (With H. E. Martin.) Arch. Ophth., **16**:733-761 (Nov.) 1936.
28. Defective central vision following successful operations for detachment of the retina. Tr. Am. Acad. Ophth., **41**:456-468, 1936; Am. J. Ophth., **20**:591-598 (June) 1937.
29. Changes in serous detachment of the retina. Am. J. Ophth., **21**:1387 (Dec.) 1938.
30. Precancerous melanosis and diffuse malignant melanoma of the conjunctiva. Arch. Ophth., **19**:354-365 (Mar.) 1938; Ophth. Soc., Tr., **35**:45-56, 1937.
31. Operative treatment of primary glaucoma. Tr. Med. Soc. State of No. Car., **84**:274-279, 1937.
32. Intraocular tumors. Southern Surg., **7**:194-203 (June) 1938.
33. Operative treatment of radiation cataract. Arch. Ophth., **21**:476-477 (Mar.) 1939.
34. The value of early operation in chronic primary glaucoma. J.A.M.A., **113**:1204-1206 (Sept.) 1939.
35. Dr. John Martin Wheeler (obituary). Tr. Am. Ophth. Soc., **37**:22-25, 1939.
36. Unilateral exophthalmos and its surgical treatment. Penna. Med. J., Sect. Eye, Ear, Nose & Throat, **43**:605-611 (Feb.) 1940.
37. Relation of drusen of the optic nerve to tuberous sclerosis. Arch. Ophth., **24**:187-203 (July) 1940.
38. Orbital tumors and their surgical treatment. No. Car. M. J., 1:583-592 (Nov.) 1940.
39. Glaucoma surgery during the past 10 years. Surg., Gyn. & Obstet., **72**:490-498 (Feb.) 1941.
40. Orbital tumors and their surgical treatment: Part I—Am. J. Ophth., **24**:386-394 (Apr.) 1941; Part II—Am. J. Ophth., **24**:497-502 (May) 1941.
41. The treatment of retinoblastoma (retinal glioma): By a combination of surgery and radiation. (Martin.) Tr. Am. Acad. Ophth., March-April, 1941, pp. 70-97; Arch. Ophth., **27**:40-92 (Jan.) 1942.
42. Classification of ocular pathology. Arch. Ophth., **24**:1077-1099 (Dec.) 1940.
43. Recent advances in ophthalmology. Texas State J. Med., **37**:475-478 (Nov.) 1941.
44. Standardizing and checking tonometers. Am. J. Ophth., **25**:478-479 (Apr.) 1942.
45. Rhabdomyosarcoma of the orbit. (With F. P. Calhoun, Jr.) Arch. Ophth., **27**:558-578 (Mar.) 1942; Tr. Am. Ophth. Soc., **39**:139-161, 1941.
46. Relation of field contraction to blood pressure in chronic glaucoma. (With John S. McGavie.) Arch. Ophth., **27**:845-850 (May) 1942.
47. Pigmented tumors of the eye and adnexa. Am. J. Ophth., **25**:1122-1124 (Sept.) 1942.
48. Exophthalmos associated with thyroid disease. N.Y. State J. Med., **42**:1848-1850 (Oct.) 1942.

49. Precancerous melanosis and the resulting malignant melanoma (cancerous melanosis) of conjunctiva and skin of lids. Tr. Am. Ophth. Soc., **40**:62, 1942; Arch. Ophth., **29**:737-746, 1943.

50. Unusual manifestations of malignant melanoma of uveal tract. Soc. Tr. N.Y. Acad. Med., April 19, 1943; Arch. Ophth., **30**:288-289, 1943.

51. The eye manifestations of xeroderma pigmentosum. (With Inez E. Wilder.) Am. J. Ophth., **26**:901-911, 1943.

52. Endophthalmitis due to B. subtilis following injury. (With Deborah Khorazo.) Am. J. Ophth., **26**:1251-1253, 1943.

53. Pigment freckles of the iris (benign melanomas): Their significance in relation to malignant melanoma of the uvea. Am. J. Ophth., **27**:217-225 (Mar.) 1944.

54. Congenital cataract following German measles in the mother. Am. J. Ophth., **27**:483-487 (May) 1944.

55. Deep-chamber glaucoma due to the formation of a cuticular product in the filtration angle. Tr. Am. Ophth. Soc., **42**:155-169, 1944; Am. J. Ophth., **27**:1193-1205 (Nov.) 1944.

56. Partial resection of the lid and plastic repair for epithelioma and other lesions involving the margin of the lid. Arch. Ophth., **32**:173-178 (Sept.) 1944.

57. The participation of the eye in general diseases. Southern Med. & Surg., **107**:61-67 (Feb.) 1945.

58. The eye in systemic diseases. Modern Med., **13**:114-115 (May) 1945.

59. Treatment of bilateral retinoblastoma (retinal glioma) surgically and by irradiation. Arch. Ophth., **33**:429-438 (June) 1945.

60. Persistence and hyperplasia of the primary vitreous (tunica vasculosa lentis or retrolental fibroplasia). Am. J. Ophth., **29**:1-19 (Jan.) 1946.

61. The iridencleisis operation for glaucoma. Arch. Ophth., **34**:360-366 (Nov.-Dec.) 1945.

62. The changing scope of medical specialties. Am. J. Ophth., **29**:1592-1594 (Dec.) 1946.

63. Retrolental fibroplasia. Sight-Saving Rev., **16**:123-126 (Fall) 1946.

64. Pigmented tumors (deSchweinitz Lecture). Am. J. Ophth., **30**:537-565 (May) 1947.

65. Pigmentation of the palpebral conjunctiva resulting from mascara. Tr. Am. Ophth. Soc., **44**:113-116, 1946.

66. The significance of the base pressure in primary glaucoma. Am. J. Ophth., **31**:25-27 (Jan.) 1948.

67. The use of X-ray therapy for retinal diseases characterized by new-formed blood vessels (Eales' disease, retinitis proliferans): A preliminary report. (With Jack S. Guyton.) Tr. Am. Acad. Ophth., May-June, 1947.

68. Retrolental fibroplasia. Am. J. Ophth., **31**:623-625 (May) 1948.

69. Invasion of the optic nerve by retinoblastoma. Arch. Ophth., **40**:553-557 (Nov.) 1948.

70. Treatment of bilateral retinoblastoma by irradiation and surgery: Report on 15-year results. (With G. R. Merriam and H. E. Martin.) Am. J. Ophth., **32**:175-190 (Feb.) 1949.

71. The IV Argentine congress of ophthalmology. Am. J. Ophth., **32**:454-456 (Mar.) 1949.

72. Persistence and hyperplasia of primary vitreous: Retrolental fibroplasia. Two entities. Arch. Ophth., **41**:527-549 (May) 1949.

73. Retrolentale fibroplasien. (With F. C. Blodi.) Klin. Monatsbl. f. Augenh., **114**:18-24, 1949.

74. Herniation of the anterior hyaloid membrane following uncomplicated intracapsular cataract extraction. Tr. Am. Ophth. Soc., **46**:73-96, 1948; Am. J. Ophth., **32**:933-940 (July) 1949.

75. Fibroplasia retrolenticular. Anal. de la Soc. Mex. Oftal., **22**:207-215 (Oct.-Dec.) 1948.

76. Heredity and retinoblastoma. Arch. Ophth., **42**:119-122 (Aug.) 1949.

77. Retrolental fibroplasia. Ophthalmologia Ibero Americana, **11**:18-28, 1949.

78. Persistence and hyperplasia of primary vitreous: retrolental fibroplasia. Two entities. A.M.A. (Sec. of Ophth.) 1948, pp. 36-64.

79. Spontaneous cysts of the ciliary body simulating neoplasms. Tr. Am. Ophth. Soc., **47**:138-146, 1949; Am. J. Ophth., **33**:1738-1746 (Nov.) 1950.

80. Retinal dysplasia. Am. J. Ophth., **33**:23-32 (Jan.) 1950.

81. Retrolental fibroplasia: A hazard of premature birth. (With H. Speert and F. C. Blodi.) Am. J. Obstet. & Gynec., **59**:246-258 (Feb.) 1950.

82. Retrolental fibroplasia. Sight-Sav. Rev. Spring, 1950.

83. Retrolental fibroplasia (Proctor Lecture). Am. J. Ophth., **34**:1-24 (Jan.) 1951.

84. Persistence and hyperplasia of primary vitreous: Retrolental fibroplasia. Two entities (abstract). Zentralbl. f. Ophth., **52**:267 (Apr.) 1950.

85. Present status of retrolental fibroplasia. Sight Sav. Rev. **21**: Summer, 1951.

86. Invasion of the optic nerve by retinoblastoma. Zentralbl. Neuro. & Psych., **111**:289 (Jan.) 1951.

87. Fibroplasia retrolental. Actas del IV Congreso Argentino de Oftalmologia, Mar del Plata, December 13-18, 1948.

88. Treatment of bilateral retinoblastoma by irradiation and surgery. Actas del IV Congreso Argentino de Oftalmologia, Mar del Plata, December 13-18, 1948.

89. Retinal dysplasia. (With F. C. Blodi.) Ber. u. d. allgem. u. spez Pathologie **8**:189-190 (May) 1951.

90. Retrolental fibroplasia. (With F. C. Blodi.) Modern Medicine, **19**:108-113 (June) 1951.

91. Retrolental fibroplasia. (With F. C. Blodi.) Acta XVI Conc. Ophthal. (Britannia) 1950.

92. Clinical and pathological characteristics of radiation cataract. (With David G. Cogan and David D. Donaldson.) Arch. Ophth., **47**:55-70 (Jan.) 1952.

93. Results of use of corticotropin (ACTH) in treatment of retrolental fibroplasia. (With Frederick C. Blodi, John C. Locke, William A. Silverman, and Richard L. Day.) Arch. Ophth., **47**:551-555 (May) 1952.

94. Association of uveal nevi with skin nevi. Arch. Ophth., **48**:271-275 (Sept.) 1952.

95. The pathology of early retrolental fibroplasia: With an analysis of the histologic findings in the eyes of new-born and stillborn infants. (With F. C. Blodi and J. C. Locke.) Am. J. Ophth., **35**:1407-1426 (Oct.) 1952.

96. Incidence of retrolental fibroplasia in a New York nursery. (With W. A. Silverman, F. C. Blodi, J. C. Locke, and R. L. Day.) Arch. Ophth., **48**:698-711 (Dec.) 1952.

97. Retrolental fibroplasia (symposium). (With F. C. Blodi.) N. Y. State J. Med., **52**:2869-2872 (Dec.) 1952.

98. Focal scleral necrosis. (With I. S. Jones.) Arch. Ophth., **49**:633-636 (June) 1953.

99. Hemangioma of the eye and adnexa. Tr. Ophth. Soc. Australia, **12**:187-191, 1952.

100. Cataract extraction. Tr. Ophth. Soc. Australia, **12**:50-55, 1952.

101. Pigmented lesions of the conjunctiva. Tr. Ophth. Soc. Australia, **12**:56-68, 1952.

102. Primary glaucoma. Tr. Ophth. Soc. Australia, **12**:43-49, 1952.

103. A classification of retrolental fibroplasia. (Nat. Soc. Preven. Blind. Publ. #193.) Am. J. Ophth., **36**:1333 (Oct.) 1953.

104. Hematopoiesis in and around the eye Am. J. Ophth., **38**:214-221 (July Pt. II) 1954.

105. Occurrence of cystoid spaces in the lens. (With Joseph A. C. Wadsworth.) Arch. Ophth., **51**:315-317 (Mar.) 1954.

106. Frequency of retinoblastoma in the progeny of parents who have survived the disease. Arch. Ophth., **52**:815 (Dec.) 1954.

107. Cicatricial stage of retrolental fibroplasia. (With J. Stepanik.) Am. J. Ophth., **38**:308-316 (Sept.) 1954.

108. Prof. Maximilian Salzmann (obituary). Arch. Ophth., **52**:808-809 (Nov.) 1954.

109. Symposium: Retrolental fibroplasia (retinopathy of prematurity). Tr. Am. Acad. Ophth., Jan.-Feb., 1955.

110. Precancerous and cancerous melanosis of the conjunctiva. Am. J. Ophth., **39**:96-100 (Apr.) 1955.

111. Treatment of retinoblastoma by radiation and triethylene melamine. Arch. Ophth., **53**:505-513 (Apr.) 1955.

112. Persistent hyperplastic primary vitreous: The Jackson Memorial Lecture. Tr. Am. Acad. Ophth., May-June, 1955; Am. J. Ophth., **40**:317-333 (Sept.) 1955.

113. An epitaph for retrolental fibroplasia. Am. J. Ophth., **40**:267 (Aug.) 1955; Sight-Sav. Rev., **25**:204-206 (Winter) 1955.

114. Pathology: Presidential Address. Tr. Am. Acad. Ophth., Sept.-Oct., 1955.

115. The treatment of expanding lesions of the orbit (Bedell Lecture). Am. J. Ophth., **41**:3-11 (Jan.) 1956.

116. Telangiectasis of the retina and Coats' disease (Gifford Lecture). Am. J. Ophth., **42**:1-8 (July) 1956.

117. Benign melanomas of the retinal pigment epithelium. (With I. S. Jones.) Am. J. Ophth., **42**:207-212 (Aug.) 1956.

118. Metaherpetic keratitis. Am. J. Ophth., **42**:489 (Sept.) 1956.

119. Combination therapy of retinoblastoma with triethylene melamine and radiotherapy. J.A.M.A., **162**:1368-1373 (Dec.) 1956.

120. Medullo-epithelioma (dictyoma) of the optic nerve. Am. J. Ophth., **44**:4-6 (July) 1957.

121. The treatment of retinoblastoma by radiation and triethylene melamine. Am. J. Ophth., **43**:865-872 (June) 1957.

122. Problems encountered in an eye clinic for children. Am. J. Ophth., **43**:24-27 (Jan.) 1957.

123. The differential diagnosis of malignant melanoma of the choroid. (With I. S. Jones.) Arch. Ophth., **58**:477-482 (Oct.) 1957.

124. Exenteration of the orbit with transplantation of the temporalis muscle. Am. J. Ophth., **45**:386-390 (Mar.) 1958.

125. Retinal dysplasia. (With B. R. Straatsma.), Am. J. Ophth., **45**:199-211 (Apr., Pt. II) 1958.

126. Optic neuritis following cataract extraction. Read before the Am. Acad. Ophth. October, 1957. Am. J. Ophth., **45**:659-662 (May) 1958.

127. The adhesion of the lens capsule to the hyaloid membrane and its relation to intracapsular cataract extraction. (With J. A. C. Wadsworth.) Tr. Am. Ophth. Soc., **55**:239-248, 1957; Am. J. Ophth., **46**:495 (Oct.) 1958.

Books

Tumors of the eye. New York, Paul B. Hoeber, Inc., 1951

Lectures

deSchweinitz Lecture (1947) ....Pigmented tumors.
Proctor Lecture (1951) ....Retrolental fibroplasia.
Jackson Lecture (1955) ....Persistent hyperplastic vitreous.
Bedell Lecture (1956) ....The treatment of expanding lesions of the orbit.
Gifford Lecture (1956) ....Telangiectasis of the retina and Coats' disease.
Snell Lecture (1956) ....Pigmented tumors and simulating lesions of the eye.

Contributor

The relation of prematurity to ocular anomalies in postnatal development. Chapter 2, pp. 12-23, *Systemic Ophthalmology.* Arnold Sorsby, Editor. London, England, Butterworth & Co. Ltd., and St. Louis, C. V. Mosby Co., 1951.

Anatomy of the retina and the optic nerve. Chapter 6, pp. 53-60, *The Eye and Its Diseases.* Conrad Berens, Editor. Philadelphia, Saunders, 1949.

Tumors. Chapter in *Ophthalmology in the War Years*—1940-1943. Chicago, Year-Book, pp. 1061-1074.

Treatment of tumors of the eye. Chapter 127, pp. 2137-2151, *The Treatment of Cancer and Allied Diseases.* George T. Pack and Edward M. Livingston, Editors. New York, Hoeber, 1940.

The eyeball. Chapter in *Cyclopedia of Medicine.* Philadelphia, Davis, 1936.

Fascicles

Tumors of the eye and adnexa. Atlas of Tumor Pathology. Section X, Fascicle 38. Armed Forces Institute of Pathology, Washington 25, D.C.

In press

Neoplasms. Chapter 14 in *Pediatric Ophthalmology.* T. S. Sanders, editor. St. Louis, Mosby.

Culture of uveal melanomas. (With G. Ehrlich.) Read before the Association for Research in Ophthalmology, June 1958. To be published in the Am. J. Ophth. (See this issue, page 163.)

The treatment of retinoblastoma by x-ray and triethylene melamine. (With George Hyman, N. Tapley, and A. W. Forrest.) Read before the Eye Section, A.M.A., June, 1958.

SERENDIPITY*

Remarks made on acceptance of the Proctor Medal

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New York

It has been said that when a man gets old you should either shoot him or pin a medal on him. I am glad that you chose the medal. I am not sure, however, that my wife feels this way. Since I was notified that I would be the recipient of this much coveted medal, I have been regaling her about the tremendous honor that is implied; perhaps I have been rather difficult to live with. At one of my sessions extolling the great honor I said, "You know, dear, there are not many great men in the world, are there?" Her reply was, "I don't know how many, but I do know that there is one less than you think."

According to a recent editorial in the *Transactions* of the American Academy of Ophthalmology and Otolaryngology the Friedenwald Lecturer is recognized for what he is doing, the Proctor Medalist for what he has done. Recounting the galley proofs I have corrected and returned of late, I couldn't help but think that I and the sea squid have something in common. As he courses backward he throws out a screen of black ink.

I remember vividly the first dinner at which this medal was awarded. It was given to our beloved Jonas Friedenwald. My recollection is that he said clinical research had some virtue. Nevertheless, I concluded that this medal was and always would be beyond my grasp. In the past the honor has been

* From the Institute of Ophthalmology of The Presbyterian Hospital of the City of New York.

given only in recognition of basic research work. In this category I certainly do not qualify for my only contribution to this field is Rhesus (Reese's) monkey. You have, however, graciously seen fit to recognize the clinician who attempts research work and as representative of this group I accept it. The basic research of you titans of medicine is to clinical research as is the hen to the pig in a little story I remember. As they were walking down the street, the hen and the pig noticed that in every lunchroom people were eating ham and eggs. The hen suggested to the pig that they go into business together. The pig demurred on the premise that for the hen the work was routine but for him it was a sacrifice.

Clinical research is notorious for its handicaps and, of course, the one most often mentioned is "lack of controls." Twins were born to an irreligious father and a very pious mother. These poor parents could not agree on whether the twins should be baptized. They compromised, therefore, baptizing one and keeping the other as a control. The difficulty with controls is bad enough even in biologic experiment. In clinical investigation the controllable features of the experiment vary from day to day, even disappear altogether, so that sometimes nothing is left but the wish of the investigator. We are often stranded, therefore, with no controls but with only our enthusiasm and our critique; too frequently the former prevails. No wonder it has been said that half of medicine is wrong and the other half is debatable. Small surprise that one professor of medicine, after teaching his students all the medicine he knew, told them at graduation: "Now that you have finished your course, I want to tell you one more thing: Half of all I have taught you is wrong, but the trouble is I do not know which half." With so much untenable or debatable we are thus led into faulty logic and reasoning.

In spite of its shortcomings clinical research has some important discoveries to its credit. The trigger mechanism of some of the great advances in medicine has resulted from accurate observation. It remains, of course, for the laboratory to explain and elucidate such observation and to give us the bulk of our present-day medical knowledge. Many of these initial observations, made not only by doctors but even by laymen, have led to fundamental research being directed along lines which have opened up entirely new vistas in medicine.

Discoveries are seldom made through logic and reason for in the natural sciences there are too many variables, inconstants, and tenuous data. Knowledge is so incomplete that at best we can only reason on probabilities and possibilities. Discoveries are made usually by the alert prepared mind unwilling to discard observations if they happen to be inconsistant with accepted doctrines.

In the vein that discoveries are not foreseen, come about unexpectedly, contain an element of chance, and are not in accord with current belief, Alan Gregg wrote, "One wonders whether the rare ability to be completely attentive to and to profit by nature's slightest deviation from the conduct expected of her is not the secret of the best research minds and one that explains why some men turn to remarkably good advantage seemingly trivial accidents. Behind such attention lies an unremitting sensitivity."

Over the entrance to the Harvard Medical School there is this inscription "Chance favors the prepared mind." We might add to this "intuition." A mind saturated with a subject may have some law and order emerge from the subconscious mind and flashes such a revelation to the conscious mind as an intuition. A striking instance of this was when Kekulé was riding on a London bus and suddenly, like a bolt out of the blue, he envisioned the benzene ring.

Schiller states "The slowness and difficulty with which the human race makes discoveries and its blindness to the most obvious facts, if it happens to be unprepared or unwilling to see them, should suffice to show that there is something gravely wrong about the logician's account of discovery."

Wilfred Trotter says that reason not only

has few discoveries to its credit compared to empiricism but often reason has obstructed the advance of science owing to false doctrines based on it.

Beveridge states, "In medicine, particularly, practices founded on reason alone have often prevailed for years or centuries before someone with an independent mind questioned them and in many cases showed they were more harmful than beneficial. The discovery is illogical but its development and expansion is logical."

Major advances in medicine have been made in the following categories:

1. By those doing basic or pure research when discordant or seemingly irrelevant notes appeared in their experiments. Often they have been working on a hypothesis which in itself proved incorrect but some unexpected tangent became the important issue. Usually, however, basic research has had to do with clarifying, consolidating, and amplifying discoveries.

2. By those doing applied or clinical research when they have been receptive to the unmasking of nature or have appreciated the significance of resemblances or recurrences.

3. By laymen who have recognized a cause and effect relationship. Sometimes this has come from individuals and at other times from antiquity as lore.

Illustrations

Illustrations of discoveries where chance, empiricism, and intuition have figured prominently in contrast to logic and reason will now be cited:

Chemotherapy

Ehrlich worked with aniline dyes on the hypothesis that for a drug to be effective against an organism it must have an affinity for this particular organism. He discovered that salvarsan (606) was specific against syphilis. Domagk, working with azo-dyes on the same theory, discovered that prontosil was specific against streptococci. In both instances this hypothesis was incorrect for it was shown later that in the one case it was arsenic and in the other case it was sulphanilamide that was effective and not the combining power of the dye. Arsenic and sulphanilamide had been known for years but their therapeutic qualities were unappreciated until by chance they were discovered when testing a hypothesis which was wrong. Thus, chemotherapy had its birth.

Beveridge states, "Two other equally famous chemotherapeutic drugs were discovered only because they happened to be present as impurities in other substances which were being tested. Scientists closely associated with the work have told me the story of these two discoveries but have asked me not to publish them as other members of the team may not wish the way in which they made the discovery to be made public."

Antibiotics

Fleming's appreciation of the importance of an observation made during the course of other work is of course epochal. However, the element of happenstance in the discovery of penicillin is all the more remarkable when one realizes that the particular mold on which the observation was made is rather rare and so far a diligent and wide search has not revealed one as good. Although the same observation regarding molds had been made before and after Fleming, 10 years elapsed before Florey applied the discovery under the impetus of the impending World War II.

Even from antiquity a family remedy handed down through almanacs and the like has been moldy cobwebs for festering wounds. And so began the miracle of the antibiotics.

Insulin

Professors von Mering and Minkowski, while studying the function of the pancreas, noticed that the urine of pancreatectomized dogs attracted swarms of flies and this led to the discovery that the urine was high in sugar content. Likewise, the discovery that

alloxan caused necrosis of the islet tissue of the pancreas also occurred by chance. Thus we have the developments which led to the subsequent control of diabetes by insulin.

IMMUNOLOGY

1. The principle of immunization by attenuated pathogens was discovered by Pasteur because of an unusual development in his researches on fowl cholera. After his summer vacation he found that all of his cultures were sterile. He attempted to revive them by subinoculation into fowls. The birds were not affected. These birds along with some new ones were inoculated with fresh cultures. To his surprise the previously used fowls withstood the inoculation with the fresh cultures but the new fowls succumbed after the usual incubation period. So began the use of vaccines.

2. The agglutination of bacteria by antiserum was discovered by Dr. H. E. Durham quite unexpectedly and not anticipated by any hypothesis. The usual equipment was not available so for testing a diagnostic reaction between culture and serum they used test tubes as a substitute. In one test tube which had been standing for a while the macroscopic and microscopic evidence of the phenomenon of sedimentation was noted. This occurred incidentally in the course of another investigation.

3. The same is true of Weil and Felix's identification of the O and H antigens and antibodies which have been milestones in serology.

4. Induced sensitization, or anaphylaxis, was discovered accidentally by Charles Richet when an extract of the tentacles of a sea anemone was being tested on laboratory animals to determine the toxic dose when he noted that a small second dose often proved fatal.

5. Vaccination was established as an accepted immunologic measure after a surprising long sequence of observations.

History has it that 1,000 years B.C. the custom in China was to insert material from small pox lesions into the nose of children (variolation). This practice was introduced into England about the middle of the 18th century.

Furthermore, in England it was recognized that if a person contracted a cow-pox sore from the cow's udder it produced immunity to small pox. Jenner's first report advocating vaccination was rejected for publication and it took him 40 years against great resistance to establish the principle.

VITAMINS

The vitamin story is replete with empiricism:

Vitamin A. A pharaoh suffered from night blindness and one of his high priests argued that he should eat the food of a cat which sees so well in the dark. After liver and the oils of the livers of the fish of the Nile cured the Pharaoh he ordered the court's scribes to place a scroll on the city's gates reading "Be it known that for all who suffer dimness of vision at night to eat the livers of animals and fishes will cure them."

About 1730, Norwegian fishermen and farmers discovered the virtues of cod liver oil.

In 1840 Steinhaeuser found that it cured specifically rickets and then it languished in obscurity for 80 years until it was dignified as a member of the vitamin family rich in vitamins A and D.

Vitamin B

Thiamine. Dr. Christian Eijkman, serving in the Dutch Military Hospital in Sumatra, took cognizance of the observation that beriberi, a disease which killed more Dutch soldiers than war, did not affect natives but only white settlers or soldiers, a fact known for generations. After seeing a kitchen attendant feeding chickens ordinary crude, unpolished rice he was prompted to quiz the kitchen personnel and found that the Dutch soldiers and white settlers ate washed and polished rice. As soon as his sick soldiers were fed crude rice they got well. For this ob-

servation Dr. Eijkman received the Nobel Prize.

Niacine. Dr. Joseph Goldberger, an expert in preventive medicine, was sent by the U. S. Public Health Service to the south of the United States in 1914 to study the supposedly infectious disease, pellagra, which was in epidemic form with a death rate of 70 percent of those affected. After he failed to identify the deadly microbe he was terminating his unsuccessful efforts in temporary headquarters in an orphanage where the children from six to 12 years ate grits, molasses, and over-cooked greens while those over 12 years with heavy chores were given milk and meat four times a week. In the younger group all were sick with pellagra but one, and that one was caught by Dr. Goldberger stealing milk and meat from the kitchen late one night, a practice that had been going on for a long time. From this clue Dr. Goldberger carried out experiments on volunteers and pellagra was no longer a problem.

B_{12}. The observation was made that liver corrected pernicious anemia long before the active ingredients were isolated and the treatment made secure, and thus a Nobel Prize for Minot and Castle.

Vitamin C

Ascorbic acid. In 1657 William Shakespeare's son-in-law, Dr. John Hall, described a cure for scurvy which consisted of a brew concocted of grass and water cress. Even before that Dr. William Harvey suggested that the disease could be cured and prevented by lemons.

Even though 10,000 sailors died, and it was known that the disease improved miraculously whenever ships made port and the men ate fresh fruit, vegetables, and shell fish, only to reappear once more on the long voyage home, it was not until 1772 that Captain James Cook, on his three-year voyage to the South Seas, made an infusion of barley and lime juice mandatory. Then limes for the Limeys who departed from the Limehouse became routine.

Vitamin C and iron. An old woman was accused of being a witch because her daughter had rosy cheeks and the other girls in the village were pale and their skin greenish. She was acquitted by telling how she had read in an old book the following: "Treatment for the green sickness and the disease of the pale ears is two-fold. First, soak rusty iron shavings over night in a quantity of vinegar water and in the morning drink the water. Next, into a sour apple stick several rusty nails and let them remain for a day and then throw away the nails and eat the fruit which contained them."

The rusty nails gave the iron and the vinegar and sour apple gave vitamin C, the essentials for building red blood cells and thus the cure for nutritional anemia, chlorosis or "green sickness."

Bacteriology

1. The discovery of the phenomenon of hem-agglutination by Hirst, and independently by McClelland and Hare, was made because by accident fluid containing the virus got mixed with red blood cells which became agglutinated. Later it became known that other investigators had observed this but were not alert enough to pick up the clue and visualize its significance.

2. Likewise, chance figured prominently in the discovery by Dr. Harold Cox that the yolk sac of embryonated eggs inoculated with rickettsia was an easy way of growing this virus. This revolutionized the study of many diseases and made the development of certain vaccines possible.

3. The Danish physician, Dr. C. Gram, discovered his famous stain known by his name for distinguishing different bacteria in a fortuitous way. He was trying to develop a double stain for kidney sections. He was trying to stain the nuclei violet with gentian violet and the tubules brown with iodine when he noted that the tissues were rapidly decolorized by alcohol but that certain bacteria remained a blue-black.

4. The acid-fast method of staining tubercle bacilli was discovered because Ehrlich

left some preparations on a stove which was later inadvertently lighted by someone else. By accident, therefore, the heat proved to be just what was required to stain the waxy-coated bacteria.

Fever therapy

Wagner-Jauregg noted that patients with syphilis of the central nervous system showed improvement if they contracted malaria. Thus the beginning of fever therapy which has figured in almost every branch of medicine including ophthalmology.

Electrolytes

It was noted that miners who used salt in their beer did not get heat stroke. This seems to be the first appreciation of the importance of electrolytes.

Drugs contributed by antiquity as homely remedies, or lore

1. South American Indians gave us the following:

a. *Cinchona bark* (*quinine*). This was introduced into Europe by missionaries returning from Peru.

It is said that the Indians of the Eastern United States used the bark of flowering dog-wood for fever; the colonists used it specifically for malaria; and the Confederacy used it for malaria during the Civil War when there was a blockade of the cinchona bark from South America. Later is was shown to contain the same active ingredient as cinchona bark.

Later, the discovery of atabrin, without which we could not have won the Pacific War, had a large fortuitous element.

Furthermore, it is said that 2,000 years ago the Italians knew that mosquitoes spread malaria but this was only established by scientific methods about 50 years ago.

After giving quinine to a patient with malaria who also had a fibrillating heart, Wenckebach noted that the heart condition improved. From this chance observation quinidine has become the accepted therapy for auricular fibrillation.

b. *Cocaine*. Kohler, as a student from the United States in Vienna, noted that it made his tongue numb when he tasted it. This was the beginning of local anesthesia.

c. *Curare*. Indians used this on arrow heads to paralyze their prey long before science told us it interferes with the nerve impulse by blocking the synapes.

d. *Iodine in thyroid function*. Indian medicine men high in the Andes journeyed to the coast to get sea weed and salt water fish for they knew that their soil lacked something furnished by the sea which prevented "big-neck sickness." We did not find out for a long time that iodine was the culprit in our goitre belt.

2. North American Indians are mostly on the debit side for to them are attributed the introduction of tobacco and syphilis to Europe. Their comparative sparsity of contributions is no doubt due to the fact that when North America was discovered there were only 500,000 Indians compared to 9,000,000 in South America.

3. China has given us ephedrine (from the herb ma huang) known 5,000 years ago.

4. India used the herbe rauvolfia serpentina from which the alkaloid reserpine was extracted and thus the tranquilizers.

5. England is accredited with digitalis when a spinster lady noted that swelling of the ankles disappeared when foxglove was chewed.

Miscellaneous

1. Dr. Sidney Ringer developed through happenstance the solution which bears his name and which has contributed so much to physiology when he noted that his frog's heart continued to beat for a much longer time than was customary. It was discovered that his laboratory assistant had used tap water instead of distilled water to make up his saline solution and this gave the clue which made it possible to determine what salts in the tap water were responsible for the increased physiologic activity.

2. Roentgen's discovery of X rays was due to a chance observation while experi-

menting with electrical discharges in high vacua, with his objective, the detection of invisible rays. Roentgen stated, "I found by accident that the rays penetrated black paper."

3. The Jews evidently realized that circumcision before the eighth day caused undue hemorrhage, and this fact is reflected in their religious laws. In recent years the laboratory told us that prior to the eighth day the newborns have a hypoprothrombinemia.

4. Our fortuitous discoveries were tryptophane by Hopkins and the recognition of the Rh factor.

Ophthalmology

The discovery, by Sir Norman Gregg, that German measles may be responsible for congenital cataracts, congenital heart disease, and other anomalies was based on an observation by one sufficiently alert and receptive to appreciate the association.

Empiricism played a large part in the application of di-iso-propyl fluorophosphate and Diamox to the glaucoma problem.

Scheie was sufficiently alert, when unusal occurrences took place in his glaucoma surgery, to give us the goniopuncture operation and the principle of scleral cauterization to promote drainage.

The cause of retrolental fibroplasia should have been established before it was. In retrospect it is easy to see that the common denominator was the incubator. Where there were incubators, there was retrolental fibroplasia. This disease was prevalent in the United States, and at first it did not appear elsewhere. When it appeared in Europe during the war it was always in the offspring of American mothers and not in the offspring of the native mothers in Germany and elsewhere. It was even branded by some as "die amerikanische Krankheit." It was found only in babies born in hospitals and not in babies born in homes. It was found in the northern part of Ireland where incubators were used, and never in the southern part of Ireland.

Finally, Campbell observed that it was never present before incubators in Australia, and of the two hospitals that she attended, she observed that in was present in the one with incubators and not in the one without incubators. Cross and Ryan observed that the disease had its entry with socialized medicine which spelled "incubators."

Also, oxygen had been viewed as not only completely innocuous but an essential for life and the more of it the better. Therefore, conditioned thinking played a role. Had something called X been administered instead of oxygen, it would have been immediately under suspicion.

And so, I think I shall conclude these illustrations on the premise that you are thoroughly convinced and thoroughly tired of hearing them, but most of all because I could not find or think up any more.

Suffice it to say, had I been so smart as the English spinster lady who discovered digitalis, or the South American Indian who found out how to prevent the big-neck sickness, I would feel more worthy of this medal.

It has been said that it is more noble to deserve a medal than to possess it. I possess this medal, but the total group of clinical researchers deserve it. We are all travelers in the land of serendipity* whether our voyages take us to laboratory or to clinic.

You probably know that this word serendipity was coined by Horace Walpole in allusion to a tale by Gauthier, "The Three Princes of Serendip," who in their travels were always discovering by chance or by sagacity things they were not in quest of or did not seek.

On very special occasions it is in order for poet laureates to come to the front. As

* "Serendipitology" has been of interest to me for years and the instances of serendipity given in this paper are in part those that I have been cognizant of over the years and in part those from other sources. One has been the fascinating book by Beveridge, *The Art of Scientific Investigation.* Another has been a pamphlet on vitamins entitled *How Vitamins have helped Man since the Dawn of History.* (Foods Plus, Inc.) Also, my erudite friend, Dr. Benjamin Rones, has been a great help.

this occasion is primarily of importance to me, I shall have to be my own poet laureate, and so:

Toss me your posies today
Whether pink, or white, or red;
I would rather have one blossom now
Than a truck-load when I'm dead.

References

Beveridge, W. I. B.: The Art of Scientific Investigation. Norton, New York, Publishers. Revised edition, 1957.

Walpole, H.: The Letters of Horace Walpole (Toynbee v. 3, 1750-1756) to Horace Mann, January, 1754.

Pamphlet: How Vitamins have helped Man since the Dawn of History. Foods Plus, Inc., publishers, 1958.

THE CULTURE OF UVEAL MELANOMAS*

The Proctor Medal Lecture

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Uveal melanomas have been enigmatic and, among others, the question has arisen, as to whether there is more than one type of melanoma encountered in the uvea. Certainly the wide cytologic spectrum seen in uveal melanomas suggests this possibility. Some tumors are composed mostly of epithelioid cells, others of branching polygonal-shaped cells, and others of spindle-shaped cells. For the most part, however, there is an intermixture of various cell types. Theobald presented evidence that some uveal melanomas arise from the ciliary nerves and were composed of Schwann cells. The idea had been advanced (Reese, 1947) that not only are there the Schwannian cell group but also a stromal cell type which has as its stem cell the melanocyte which serves as the uveal stroma. We felt that the issue might be clarified by culturing uveal melanomas and determining whether the cytologic characteristics indicate distinct types of uveal melanomas. With this objective in mind we applied to the National Institute of Health for a subsidy which was kindly granted for a period of three years. This is a report on our results.

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Material

We have cultured (1) 33 choroidal melanomas; (2) six iris and ciliary body melanomas; (3) normal uveal tissue from three eyes removed because of extraocular pathology, such as orbital or lid tumors and also routinely normal uveal tissue was cultured from the eyes harboring melanomas; and (4) 20 melanomas other than those in the uvea, such as the conjunctiva and lids. We are reporting here only on the uveal melanomas.

Method

During the first year we were concerned mostly with improvement of our technique and with methods for obtaining satisfactory growths of the tumor. We found a number of references dealing with the culture of animal and human melanomas (Grand, 1935; Grand, et al., 1935; Guldberg, 1935; Grand, et al. 1948) but only two pertaining to cultures of human ocular melanomas (Weitzmann, 1938; Attardi and Moro, 1953). At first the various accepted techniques were tried. We selected as best for our purpose the double-cover-slip, lying-drop method by Maximow (1925) modified by Murray and Stout. Undiluted chicken plasma was used as culture support and a combination of human cord serum, ox serum ultrafiltrate, and chicken embryo extract was used as feeding solution. Optimal results were obtained with

certain changes in this technique. The chicken plasma was diluted by human cord serum. At first heparinized chicken plasma was purchased but during the past two years the plasma has been prepared in our laboratory without the addition of an anticoagulant. In this way better results were obtained in cell multiplication and cellular differentiation.

When sufficient material was available 12 cultures were prepared from each specimen. Not only were the tumors cultured, but also at the same time and with the same method the distant normal part of the uvea. The remainder of the eye or the tissue was sent to our pathology laboratory for routine processing and sectioning.

The explants were selected from a nonnecrotic area of the tumor, cut into pieces measuring one to two mm. in diameter, and washed in two or three changes of sterile balanced salt solution (BSS). These fragments were planted in one drop of a culture medium composed of two parts of human placental serum and one part of chicken embryo extract (1:1) on a round coverslip which was fastened to a larger square coverslip. A drop of the same size, consisting of one part chicken plasma and two parts human placental serum, was dropped on the coverslip and, after mixing and spreading over the entire area, the fragments were oriented. The depression in the slide was inverted over the coverslip, sealed, and incubated at 36.5°C. Each culture was washed and fed two to three times a week according to its growth rate. For the washings Hanks BSS (pH 7.2) was used. The feeding solution consisted of three parts of human cord serum, one part ox serum ultrafiltate, and one part chicken embryo extract (1:1). The use of chicken plasma insures the firmness of the plasma clot and keeps the liquefaction of the plasma to a minimum. The addition of ox serum ultrafiltrate inhibits the accumulation of lipoid droplets in the cell. The tumors, and the normal tissues cultured for comparison, were kept without transfer in a healthy and active condition for a month or more. The cultures were examined daily with a microscope, and their growth and development were recorded by photomicrographs. The cultures wers sacrificed at various stages of growth and stained by different methods including Harris hematoxylin, Jenner-Giemsa, cresyl violet, Mallory's phosphotungstic-acid stain, Dopa, and Fontana.

Growth results

Growths satisfactory for study were obtained from 21 tumors, moderate but mostly inadequate growth from 15 tumors, and no growth from three tumors. The growth from the uvea of the three normal eyes was good. In general, the tumors which showed by routine hematoxylin and eosin stain to be mostly of epithelioid cells tended to have poorer culture growth than the better differentiated tumors.

Growths of malignant melanoma

(Figs. 1 through 10)

On approximately the fourth day branching pigmented cells appeared around the explant. In general their axes were concentric or haphazard to the base of the explant. In spite of the fact that mitotic figures were rarely noted we have felt that these cells represented active growth rather than an ameboid migration of cells from the explant. There seems to be no doubt but that the melanin granules in the cytoplasm were fabricated by the cell and not phagocytosed. Confirmation of this was furnished by the dopa stains (Bloch, 1929).

In addition there were also some polygonal shaped epithelioid cells. Some of these appeared to be wandering cells with perhaps phagocytosed pigment but most of them became the branched pigmented cell already described. These branching cells showed transitions to a more spindle-shaped bipolar cell, some with pigment and some without. As the branching cells became less and the long spindle-shaped cells predominated, the axes were radial to the explant. Usually the further the spindle cells were away from the

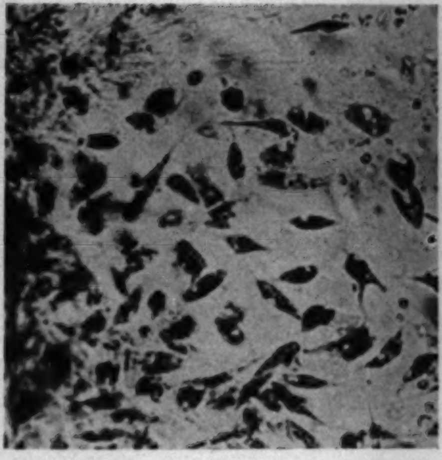

Fig. 1 (Reese and Ehrlich). Culture of malignant melanoma of the choroid. Living cells, seven days' growth. All the cells contain pigment granules in the cytoplasm. (×50.)

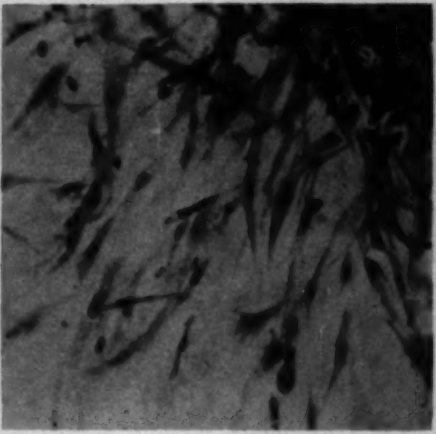

Fig. 3 (Reese and Ehrlich). Culture of malignant melanoma of choroid, 10 days' growth. The cells are becoming spindle or Schwannian in type. (Harris hematoxylin stain, ×50.)

explant the less pigment they contained.

These spindle cells answered all the criteria laid down by Murray and Stout (1940, 1942) for Schwann cells. With Mallory's phosphotungstic-hematoxylin they stained purplish, aligned themselves in formations but with individual cells adhering to one another giving the appearance of syncytia. The cells had an oval nucleus which was usually wider than the protoplasmic ribbon containing it. The nucleus was poor in chromatin and usually had one or two prominent nucleoli. They showed a hyalin threadlike cytoplasm which tended to branch at the ends. All of these characteristics are in marked

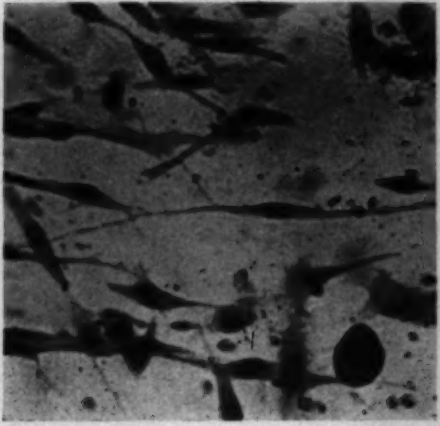

Fig. 2 (Reese and Ehrlich). Culture of malignant melanoma of the choroid, six days' growth. Mitosis. (Phosphotungistic acid hematoxylin stain, ×100.)

Fig. 4 (Reese and Ehrlich). Culture of malignant melanoma of choroid, seven days' growth. Pigmented Schwannian type of cell. (Jenner-Giemsa stain, ×100.)

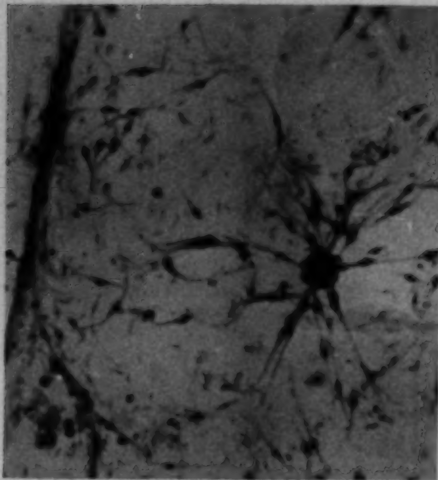

Fig. 5 (Reese and Ehrlich). Culture of malignant melanoma of choroid, seven days' growth. In the center is seen a tumor seed from which radiates a growth of typical Schwann cells. (Jenner-Giemsa stain, ×50.)

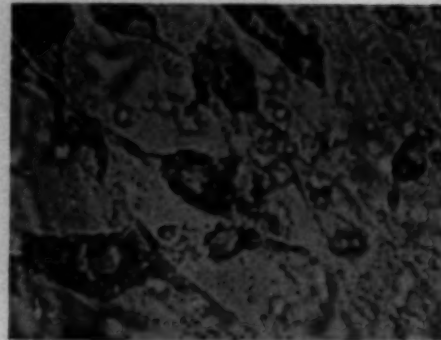

Fig. 7 (Reese and Ehrlich). Culture of malignant melanoma of iris, 11 days' growth. Living cells. Pigmented polygonal type of cell. (×200.)

contrast to those of the fibroblasts which only appear in the cultures later. It has not been possible for us to maintain the melanoma cells in continuous culture longer than

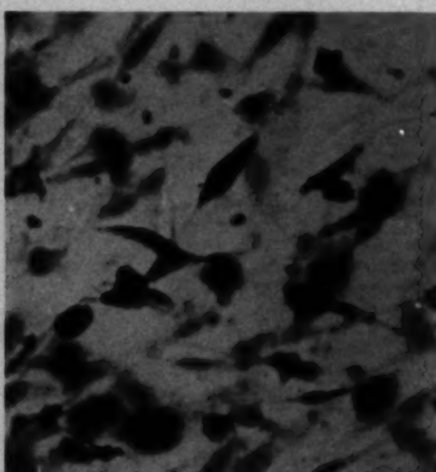

Fig. 6 (Reese and Ehrlich). Culture of malignant melanoma of the iris, 13 days' growth. Pigmented polygonal and Schwann type of cell. (Harris hematoxylin stain, ×100.)

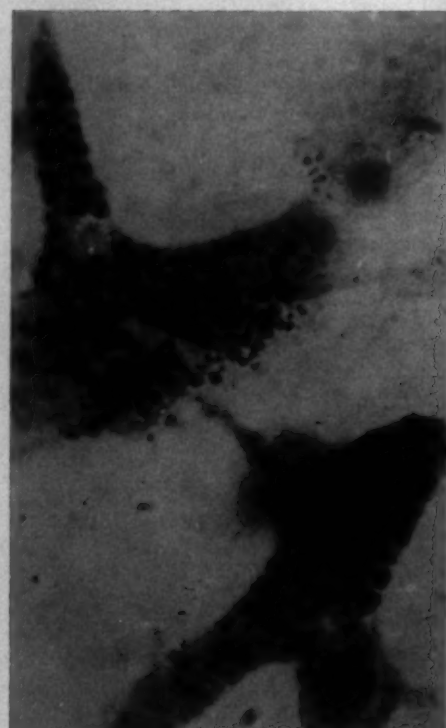

Fig. 8 (Reese and Ehrlich). Culture of malignant melanoma of the iris, 13 days' growth. Pigmented polygonal cells. (Harris hematoxylin stain, ×485.)

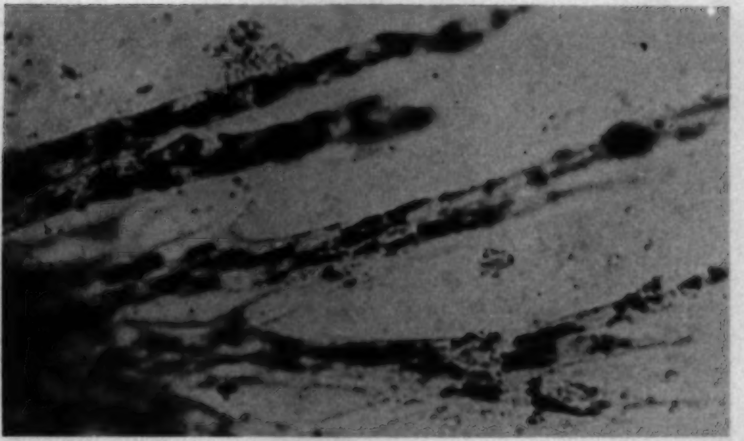

Fig. 9 (Reese and Ehrlich). Culture of malignant melanoma of the iris, 24 days' growth. Living cells. A pigmented cell peculiar to the iris interpreted as mimicking a neuroectodermal muscle cell. (×100.)

approximately one to three months.

Regardless of whether the hematoxylin and eosin stains of the tumor showed that it was for the most part epithelioid, branching, or spindle-shape in type, the growths in vitro showed the same sequence which was in the order previously mentioned of branching pigmented cells, and all stages of transition from this to the long bipolar cell interpreted as the Schwann cell. In some instances the spindle cell and the branching cell grew out from the explant at the same time but, in general, there was a metamorphosis from the branching cell to the spindle cell. The cytology of the tumor cultured did not seem to alter this order of growth.

Although in the culture growths the order of differentiation was from the branching cell to the bipolar spindle cell occasionally we saw the reverse both in the melanomas as well as the normal uvea. This apparent dedifferentiation may be seen in vivo when a spindle-cell uveal melanoma manifests an epithelioid cytology at distal metastatic sites.

Cultures from the same tumor sacrificed the same day of growth showed a great variation. One culture the same age might show delicate, branching bipolar Schwann cells and another culture from the same tumor and the same age would show the branching cells in the process of their metamorphosis to the Schwann cell.

The characteristics and behavior of the cultures of the iris melanomas were the same as those of the choroidal melanomas except in one respect. The cultures of the iris mela-

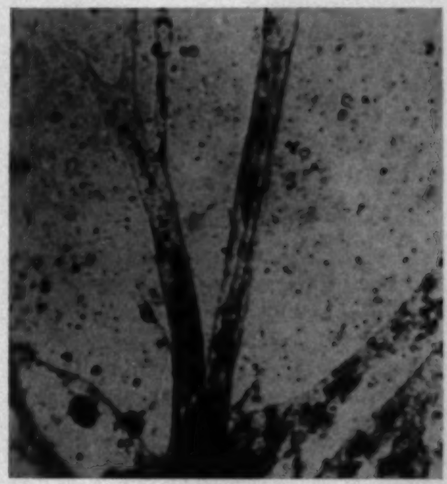

Fig. 10 (Reese and Ehrlich). Culture of malignant melanoma of the iris, four days' growth. Living cells. A pigmented cell interpreted as akin to the neuroectodermal muscle cell. (×60.)

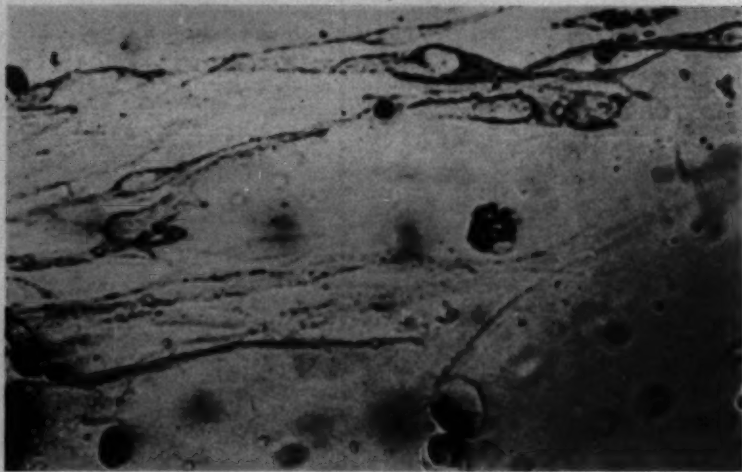

Fig. 11 (Reese and Ehrlich). Culture of normal choroid, four days' growth. Living cells. A pigmented cell approaching the Schwann cell type. (×100.) (The eye was removed because of a malignant melanoma of the choroid.)

noma as well as those of the normal iris sometimes showed a long pigmented cell with more or less parallel protoplasmic borders giving it the appearance of a fiber. These cells have been interpreted as possible rudimentary neuroectodermal muscle cells (figs. 9, 10, 19, and 20).* If so then they indicate a relationship between leiomyoma and melanoma of the iris, a contention held by Zimmerman. There was no cell in the culture which seemed to mimic the nevus cell which plays a prominent role in iris melanomas.

GROWTH OF NORMAL UVEA (Figs. 11 through 20)

The growths of what was accepted as normal uvea from eyes with uveal melanoma showed essentially the same pattern as those from the uvea of normal eyes. One of our cultures from the supposedly normal part of the uvea of an eye harboring a malignant choroidal melanoma showed an unusually florid growth. Colenbrander (1952) thought that uveal melanomas produced a growth substance which stimulated normal choroidal cells to abnormal growth. However, for the most part our cultures indicated a lag period,

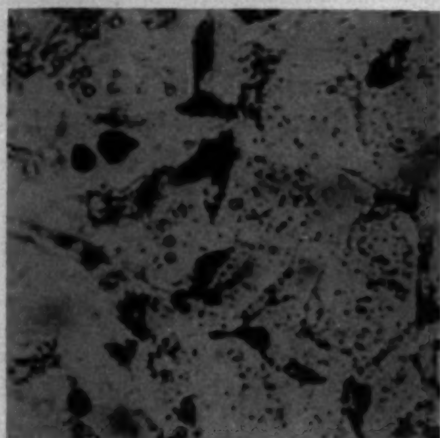

Fig. 12 (Reese and Ehrlich). Culture of normal choroid, four days' growth. Living cells. A pigmented branching cell. (×100.) (The eye was removed because of a malignant melanoma of the choroid.)

* After this paper was sent for publication, the article by Tenenbaum and Kornblueth (1958) appeared. The authors interpret the same cell as stemming from the dilator muscle layer of the iris. Their findings and ours, together with those of Rones and Zimmerman (1958), indicate that iris melanomas may have a leiomyomatous phase.

Fig. 13 (Reese and Ehrlich). Culture of normal choroid, 10 days' growth. A pigmented schwannian type cell. (Jenner-Giemsa stain, ×100.) (The eye was removed because of a malignant melanoma of the choroid.)

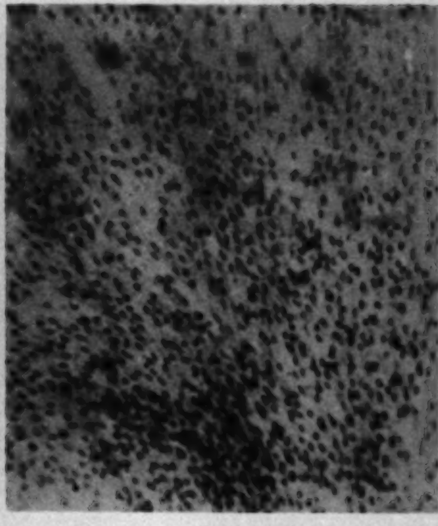

Fig. 14 (Reese and Ehrlich). Culture of normal choroid, 13 days' growth. A florid growth of spindle cells. (Jenner-Giemsa, stain, ×30.) (The eye was removed because of malignant melanoma of the choroid.)

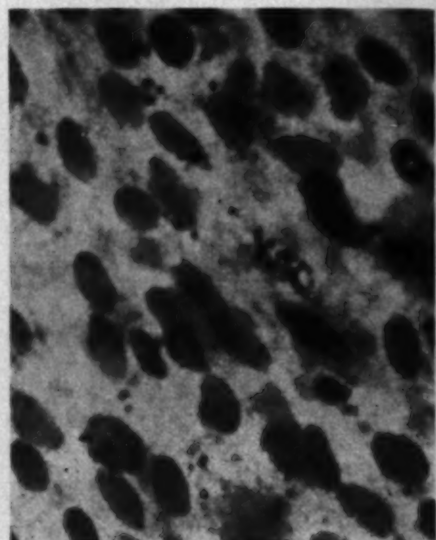

Fig. 15 (Reese and Ehrlich). Culture of normal choroid. A higher power view of the culture shown in Figure 14. (×200.)

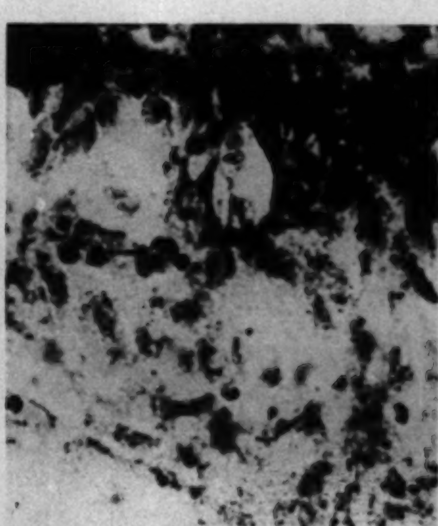

Fig. 16 (Reese and Ehrlich). Culture of normal choroid, 17 days' growth. This represents a growth of pigment epithelium. (Jenner-Giemsa stain, ×100.) (The eye was removed because of malignant melanoma of the choroid and ciliary body.)

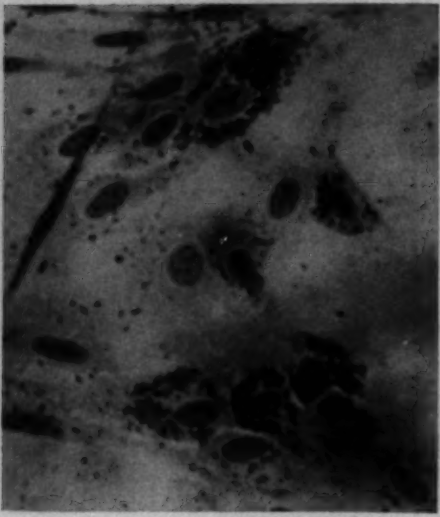

Fig. 17 (Reese and Ehrlich). Culture of normal choroid, nine days' growth. The cells represent pigment epithelium. (Jenner-Giemsa stain, ×100.) (Normal eye removed when the orbit was exenterated because of rhabdomyosarcoma.)

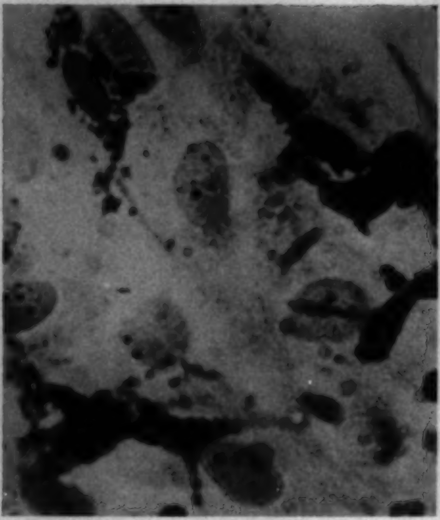

Fig. 18 (Reese and Ehrlich). Culture of normal choroid, five days' growth. A high magnification (×200) of pigment eipthelium. (Jenner-Giemsa stain.) (The eye was removed because of malignant melanoma of the choroid.)

together with a speed and character of growth the same in the normal tissue of eyes harboring melanomas as they were in those without melanomas.

We had no difficulty growing the normal uvea and the sequence of growth was the same as that in the melanomas. In general, the polygonal pigmented cells appeared first

Fig. 19 (Reese and Ehrlich). Culture of normal iris, six days' growth. Living cells. Pigmented branching cells are seen. (×100.) (The eye was removed because of malignant melanoma of the iris.)

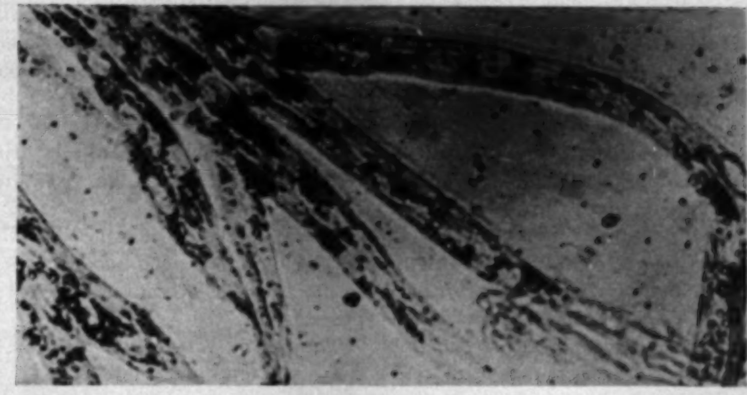

Fig. 20 (Reese and Ehrlich). Culture of normal iris, 15 days' growth. Living cells. These pigment-bearing cells seem peculiar to the iris and perhaps a rudimentary neuro-ectodermal muscle cell. It is not clear in this particular specimen whether it was from the iris or ciliary body. (×100.) (Normal eye removed when orbit was exenterated because of rhabdomyosarcoma.)

as outgrowths from the explant and then all transitional stages to the spindle cells with characteristics identical to those we associate with the Schwann cell. Occasionally this order was reversed just at it was in the melanomas whereby the Schwann cells appeared first or at least concurrently with the branching cells. We could note no great difference in the liquefaction of the media by the normal tissue and the tumor tissue.

A feature which was almost constantly present in the cultured normal uvea was the rapidly growing pigment epithelium. The growth of the pigment epithelium manifested itself early, 48 hours, outstripped all other growths, and mitotic figures were frequent. In the culture of melanomas in animal eyes Greenberg, Kopac, and Gordon (1956) found hyperplasia of the pigment epithelium a conspicuous feature and it appeared on the first day of growth. Pomerat and Littlejohn (1956) also found proliferation of the pigment epithelium a prominent feature of their cultures.

The pigment produced by the pigment epithelium in cultures is different in appearance from that seen in the cultured cells of melanomas as well as the normal cells of the uvea. The epithelial pigment is blacker and appears in small discrete dots or rods, whereas the pigment in cultures of melanomas and normal uvea is lighter brown and is amorphous and crystaloid. Different types of melanin are also suspected in animals (Ladebeck, 1922; Bohren, et al., 1943; Greenberg, Kopac, and Gordon, 1956). Gordon (1953) mentions that the melanin produced in the cultures of fowl eyes may be black, reddish, or yellow, but he thought that although the chemical differences between these three forms are not yet known each color is associated with a different type of pigment granule.

The growths of the normal iris were essentially the same as those of the choroid except for the previously mentioned long cell interpreted as a pigmented smooth muscle cell (figs. 19 and 20).

Discussion

It seems logical to assume that the pigment-bearing cells of the uvea are actually progressive stages in the differentiation of a single cell type. This cell type migrates during development from the neural crest into the future choroid—the network of vessels around the optic cup (Westerveld-Brandon, 1952; Redslob, 1925).

Bartelmez (1954) seems to be the first to observe the neural crest derivation of uveal

cells in man. Arising embryologically from the neural crest they can be viewed, therefore, as specialized nerve cells. As pointed out by Gordon, no doubt one of the main reasons why the true nature of the vertebrate pigment cell remained unknown for such a long time lies in the fact that it does not begin to differentiate, that is, to form melanin granules, until late in embryonic life and at positions quite remote from its site of origin.

The results of experimentation with amphibians, birds, and mammals are in agreement regarding an early and extensive migration of precursor (colorless) pigment cells in the embryo (DuShane, 1943; Rawles, 1948; Hoerstadius, 1950). Some of these potential melanoblasts may become located in positions unfavorable for melanin synthesis and only under pathologic conditions are they stimulated to form melanin (Gordon, 1953). It is not known whether the cells of the crest are pluripotential from the beginning and differentiate according to their later positions, or whether their potentialities are already fixed before migration begins.

The following points are in favor of the neural crest origin of the melanoblasts (Gordon, 1953; Rawles, 1948; Zimmermann, 1950):

1. Embryologic studies in amphibia, birds, mice, and human beings shows that melanocytés are derived from the neural crest region.

2. Tissue culture studies show the cells are polygonal with long, slender branching processes and, therefore, are dendritic in shape as are other nerve cells.

3. The cells can be stained with silver, gold, and methylene blue as can other nerve cells.

4. Biochemically, these cells produce the enzyme, dopa-oxidase, which makes them related in function to sympathetic nervous system cells which produce a similar compound.

5. Malignant tumors of melanocytes (malignant melanomas) do not respond to X-ray therapy and in that respect resemble most nerve-cells tumors.

6. Abnormalities of pigmentation are frequently associated with abnormalities of the nervous system, for example, neurofibromatosis.

A logical conception, therefore, of uveal melanomas seems to be that they arise from cells which have their origin from the neural crest. These are basically, therefore, neuroectodermal cells having the capabilities of developing into various cytologic types which is characteristic of neuroectodermal cells in general.

The tumors which arise from these uveal cells, therefore, may show a broad cytologic spectrum. They may be for the most part composed of one type or the other, or a combination which ranges from the epithelioid to the branching polygonal to the spindle or Schwann cell.

The epithelioid cell could be viewed as the more primitive or anaplastic, the branching polygonal or stromal type of cell as more differentiated, and the spindle or Schwann cell as the most differentiated. Therefore, the prognosis in tumors composed of the more primative cell (epithelioid) is expected to be poorer than with the more differentiated cell (Schwann). This versatility of the cell to manifest itself in various forms is characteristic of the neuroectodermal cell in general.

In tumors of the central nervous system the stem cells may be (1) glia, which may be either astrocytes or oligodendrocytes; (2) ganglion cells; (3) primitive cells (neuroblast); and (4) combination. In uveal melanoma the counterpart of the glia is the Schwann cell, of the ganglion cell is the branching or stromal cell, and of the primitive neuroblast is the epithelioid cell.

One of us (A. B. R.) postulated that uveal melanomas may arise from the stromal cell of the uvea or from the Schwann cell. These results of our cultures of uveal melanomas indicate the reason why some uveal melanomas may be composed almost entirely of some variation of the branching melanoblast, others of the epithelioid variety, and others of some type of spindle cell. Instead of hav-

ing different stem cells they have a common one in different phases of differentiation.

Summary

Cultures of uveal melanoma show a growth ranging from a branching polygonal-shaped pigment-bearing cell to a long, bipolar spindle-shaped cell with or without pigment. These latter cells have the characteristics of Schwann cells. All gradations between these two extremes can be found. Regardless of the cell type of the tumor cultured, there is the transition from the one type of cell to the other. Therefore, just as there are two principal cell types seen by conventional histology, so there are the same two types observed in culture. The presence of more than one cell type in the same tumor, is, of course, consistent with other tumors, especially those from the neuroectoderm. As uveal melanomas seem to stem from the neural crest it is not surprising, therefore, that their cells are pluripotential.

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Acknowledgment

We are particularly grateful to Dr. Lorenz E. Zimmerman for his help with the interpretation of the culture results, an aspect of this work which proved difficult. Our conclusions are in accord with views previously expressed by Dr. Zimmerman through personal communications. Also, we appreciate very much indeed the counsel from Dr. Margaret Murray and Dr. George Smelser. The photomicrographs were prepared by Edward Gonzalez, M.T. (ASCP).

References

Attardi, G., and Moro, F.: Caratteri morfologici degli elementi dei melanomi maligni della coroide coltivati in vitro. Monit. zool. ital (Firenze) **62**(Suppl.):441-445, 1953.

Bartelmez, G. W.: Formation of neural crest from primary optic vesicle in man. Contrib. Embryol. Carnegie Inst. Washington, **35**:55-72, 1954.

Bloch, B.: The problem of pigment formation. Am. J. Med. Sci., **177**:609-618 (May) 1929.

Bohren, B. B., Conrad, R. M., and Warren, D. C.: A chemical and histological study of the feather pigments of the domestic fowl. Am. Naturalist, **77**:481-518, 1943.

Colenbrander, M. C.: Mathematical diffusion of choroidal sarcoma infiltration. Nederl. tÿdschr. geneesk., **96**:1235-1237 (May) 1952.

DuShane, G. P.: The embryology of vertebrate pigment cells. Part I: Amphibia. Quart. Rev. Biol., **18**:109-127, 1943.

Gordon, M.: Pigment cell growth. Proc. 3rd conf. Biology of Normal and Atypical Pigment Cell Growth. New York Zool. Soc. New York, Academic Press, 1953.

Grand, C. G., Chambers, R., and Cameron, G.: Neoplasm studies: I. Cells of melanoma in tissue culture. Am. J. Cancer, **24**:36-50, 1935.

Grand, C. G.: Cell types found in Harding and Passey mouse melanoma grown in vitro. Proc. Soc. Exper. Biol. & Med., **32**:1196-1197 (Apr.) 1935.

Grand, C. G., and Cameron, G.: Tissue culture studies of pigmented melanomas: fish, mouse, and human. Special Publication New York Acad. Sci.: The Biology of Melanomas, **4**:171-176, 1948.

Greenberg, S. S., Kopac, M. J., and Gordon, M.: Cytology and cytochemistry of melanoma cells. Ann. New York Acad. Sci., **67**:55-122 (Oct.) 1956.

Guldberg, G.: Versuche zur Züchtung von melanotischen Geschwülsten. Arch. f. experi. Zellforsch., **17**:210-213, 1935.

Hoerstadius, S.: The Neural Crest. London, Oxford Univ. Press, 1950.

Ladebeck, E.: Die Farben einiger Hühnerrassan. Zschr. f. indukt. Abstammungs u. Vererbungsl. (Leipzig), **30**:1-62, 1922.

Maximow, A. A.: Tissue cultures of young mammalian embryos. Contrib. Embryol. Carnegie Inst. Washington, **16**:47-113, 1925.

Murray, M .R., and Stout, A. P.: Demonstration of the formation of reticulin by schwannian tumor cells in vitro. Am. J. Path., **18**:585-593, 1942.

———: Schwann cells versus fibroblasts as the origin of the specific nerve sheath tumor. Am. J. Ophth., **16**:41-60, 1940.

Pomerat, C. M., and Littlejohn, L., Jr.: Observations on tissue culture of the human eye. Southern M. J., **49**:230-237 (Mar.) 1956.

Rawles, M. E.: Origin of melanophores and their role in development of color patterns in vertebrates. Physiol. Rev., **28**:383-408 (Oct.) 1948.

Redslob, G.: Etude sur l'origine du pigment de la choroide. Ann. ocul., **162**:368-381, 1925.

Reese, A. B.: Pigmented tumors. Am. J. Ophth., **30**:537-565 (May) 1947.

Rones, B., and Zimmerman, L. E.: The prognosis of primary tumors of the iris treated by iridectomy. Arch. Ophth., 60:193-205, 1958.

Tenenbaum, E., and Kornblueth, W.: Cultivation of adult human iris in vitro. Arch. Ophth., 60:312-318, 1958.

Theobald, G. D.: Neurogenic origin of choroidal sarcoma. Arch. Ophth., 18:971-997 (Dec.) 1937.

Weitzmann, G.: Ueber die Züchtung einiger menschlicher maligner Tumoren in vitro. Ztschr. f. Krebsforsch., 48:1-20, 1938.

Westerveld-Brandon, E. R.: The nature of choroidal tumors. Nederl. tÿdschr. geneesk., 96:3043-3044, 1952.

Zimmermann, A. A.: The development of epidermal pigmentation in the negro fetus. Zoologica, 35:10-12, 1950.

Zimmerman, L. E.: Personal communications.

AN ELECTROMYOGRAPHIC STUDY OF ASYMMETRIC CONVERGENCE*

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Asymmetric convergence refers to convergence occurring in any other direction than along the median plane. In this paper, we are concerned only with a special type of asymmetric convergence in which the fixation points are restricted to the stationary visual axis of one eye. In the past few years, there have been several reports by various investigators using different techniques to determine what occurs in the apparently stationary eye during this type of asymmetric convergence, and how their findings correlate with Hering's law of equal innervation to the extraocular muscles.[1-3]

The problem is this: In Figure 1, as the right eye converges from A to B, two points along the axis of the left eye, the left eye appears to remain stationary. Yet, according to Hering's law, the left lateral rectus should receive a stimulus for levoversion, and the left medial rectus for convergence. What happens, if anything, to these innervations to the left eye to enable that eye to remain apparently stationary?

Hering[4] himself simply explained that the two innervations, one for version and one for vergence, acting in opposite directions, counteract each other, resulting in no movement of the eye on whose axis the target lies. Hering felt that both innervations actually reached the antagonistic muscles of the stationary eye rather than balancing out centrally in the brain. He even suggested that this might be proved by testing for an increase in intraocular pressure in the stationary eye. In addition, he was able to hear an increase in muscle noise in the stationary eye by means of a "noise funnel" (Schalltrichter).[5] He also described a slight quiver of the apparently stationary eye, suggesting that this oscillation resulted from different speeds of the two antagonistic innervations in reaching the eye.

Breinin[1] had observed by electromyography that whenever an eye turned, be it during a version or vergence movement, there was an increase in the electrical activity of the agonist and reciprocal inhibition of the antagonist. When he noted no change in activity in either horizontal rectus of the stationary eye during asymmetric convergence, he concluded that the antagonistic innervations for a version and vergence movement cancelled each other in the brain and never reached the muscles. He says that it must be so, for if the innervation of that eye were to change, it would have to move, but fixation precludes such movement. The

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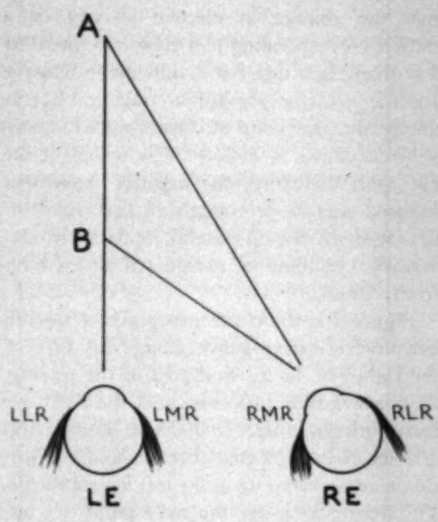

Fig. 1 (Tamler, et al.). Diagram of asymmetric convergence in which fixation points A and B lie on the stationary visual axis of the left eye.

alternative, he goes on, of an equal increase in innervation of medial and lateral rectus, does not occur. In view of the fact that Hering did not believe in central neutralization of opposing innervations and, indeed, noted slight movement of the apparently stationary eye, it appears to us that Breinin's electromyographic data do not agree with Hering's interpretation of this phenomenon. In a more recent statement on this problem, Breinin does not significantly alter his point of view.[5, 6]

Shortly after this work of Breinin, two other reports appeared utilizing other techniques, which conflicted with Breinin's ideas. Westheimer and Mitchell[2] recorded the movements of both eyes during rapid asymmetric convergence by imaging the reflections of a light source in each of the subject's corneas on a continuously moving photographic film. They noted two separate movements of both eyes. First, there is a saccadic or rapid version movement of the two eyes. This is quickly followed by a convergence movement of both eyes, bringing the visual axes in position to intersect at the new fixation point. Thus, even the apparently stationary eye swings out in unison with the version movement of the other eye, and then is brought in again during the binocular convergence response, so that it finally assumes the same direction it had at the outset. The same observations were made by Dodge[7] in 1902. Such movement indicates that the innervations reach the muscles of the "stationary" eye and that there is a peripheral adjustment rather than a central neutralization.

Alpern and Ellen[3] confirmed the findings of Westheimer and Mitchell using electro-oculography.* In binocular asymmetric convergence, they, too, found that the eye on the axis of the target makes a small rapid movement at first, but then quickly returns to its original position. They also noted similar movements in the fixating "stationary" eye when a small horizontal prism (five to 15 prism diopters) is suddenly introduced before the other eye. Their findings also indicate a peripherally manifested adjustment between vergence and version. They offer an explanation for the electromyographic failure to record change in activities in the involved muscles of the "stationary" eye by suggesting that the needle electrodes did not pick up enough electrical activity during these relatively small movements to give an observable change on the tracing.

An electromyographic study by Blodi and Van Allen[5] agrees with Breinin that there is no change in electrical potential of the horizontal muscles of the apparently stationary eye during asymmetric convergence. It is interesting to note that they do report one instance of an increase from 60 microvolts to 80 microvolts in activity of the external rectus of the stationary eye just before break of convergence. This is a change of at least 20 percent.

So, to summarize the recent literature thus

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* Electro-oculography is a technique discussed by one of us (E. M., Arch. Ophth., **45**:169, 1951) for measuring direction and amount of eye movement by the use of surface electrodes on the skin around the orbit.

[illegible]r on this subject, we find Hering's view of peripheral adjustment of opposing version and vergence innervations confirmed by the nonelectromyographic work of Westheimer and Mitchell and Alpern and Ellen. The electromyographic data to date, that of Breinin and Blodi and Van Allen, appear to be in conflict with this.

It is the purpose of this paper to present electromyographic data which support Hering's original thesis as confirmed by Westheimer and Mitchell and Alpern and Ellen.

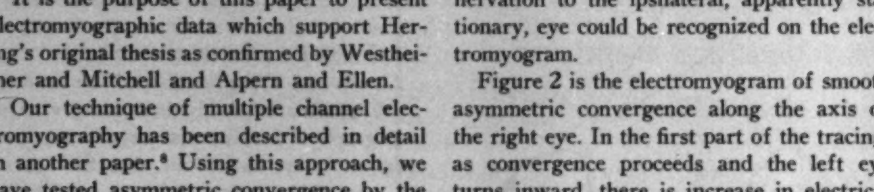
Our technique of multiple channel electromyography has been described in detail in another paper.[8] Using this approach, we have tested asymmetric convergence by the following two stimulus methods:

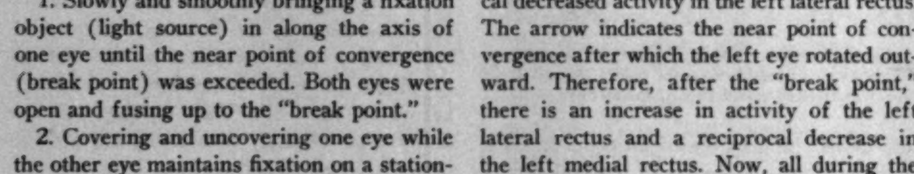
1. Slowly and smoothly bringing a fixation object (light source) in along the axis of one eye until the near point of convergence (break point) was exceeded. Both eyes were open and fusing up to the "break point."

2. Covering and uncovering one eye while the other eye maintains fixation on a stationary target.

The sensitivity of the apparatus is such that the change in electric activity of a muscle corresponding to a slow movement of less than eight degrees is difficult to observe on the electromyographic tracing. Fortunately, the near point of convergence in many subjects comes to within a few inches of the eye and, therefore, the angular movement induced was large enough so that yoke innervation to the ipsilateral, apparently stationary, eye could be recognized on the electromyogram.

Figure 2 is the electromyogram of smooth asymmetric convergence along the axis of the right eye. In the first part of the tracing, as convergence proceeds and the left eye turns inward, there is increase in electrical activity of the left medial rectus and reciprocal decreased activity in the left lateral rectus. The arrow indicates the near point of convergence after which the left eye rotated outward. Therefore, after the "break point," there is an increase in activity of the left lateral rectus and a reciprocal decrease in the left medial rectus. Now, all during the time that the left eye was converging and diverging, the right eye remained stationary.





Figs. 2 and 3 (Tamler, et al.). (Above, fig. 2). Binocular smooth asymmetric convergence along the axis of the right eye. Arrow indicates "break-point" of convergence. The right eye is stationary throughout. Note simultaneous increase in activity of horizontal rectus muscles of the right eye before "break-point" and simultaneous decrease after it. (Below, fig. 3). Another example of smooth asymmetric binocular convergence along the axis of the right eye. Arrow indicates "break-point" of convergence. The right eye is stationary throughout. Note increase before and decease after "break-point" of horizontal rectus muscles of the right eye.

Note that there is a simultaneous increase in activity of both horizontal rectus muscles of the stationary right eye as the left eye converged. Note further that when fusion is broken and the left eye diverges, there is a simultaneous decrease in activity of both horizontal recti of the stationary fixing right eye.

Figure 3 is the electromyogram of another patient, again following a target in along the axis of the right eye. Again as the left eye converges, there is simultaneous increase in electrical activity of the horizontal recti of the right eye, up to the "break point" (arrow). After that, there is again a simultaneous decrease in activity of the horizontal recti of the stationary eye as the left eye diverges.

If one covers an eye of a subject with an exo deviation during near binocular fixation, that eye will deviate outward under cover. If, while the unoccluded eye maintains fixation on the near target, one removes the cover from the deviating eye, the latter will make a fusional convergent movement while the former will appear to remain stationary. This, too, is a type of asymmetric convergence. It is necessary to select subjects with high exophoria in order to get observable electromyographic changes in the fixing eye.

In the first half of Figure 4, the left eye of a patient with 15 degrees of intermittent exotropia is under cover and turned outward while the right eye fixes a near target. The arrow indicates uncovering of the left eye. The left eye then makes a fusional convergent movement represented on the tracing by increase in activity of the left medial rectus and reciprocal decrease in activity of the left lateral rectus. The stationary right eye, continuously maintaining fixation on the target, shows simultaneous increase in activity of both its medial and lateral rectus muscles as the left eye converges to fuse. This increase is small since we are working near the limits of instrument sensitivity, but significant.

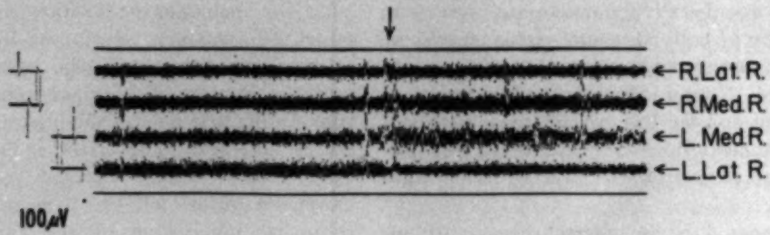
In Figure 5, we again have a fusional convergent movement of one eye while the other eye maintains its fixation on a near target. This was in a patient with 18 degrees of intermittent exotropia who could voluntarily let one eye diverge and converge to fuse. In the first half of this figure, the left eye is diverging. The arrow indicates the start of a voluntary fusional convergent movement of the left eye with subsequent reciprocal innervation of the appropriate horizontal muscles of that moving eye. As the left eye converges, the stationary fixing right eye shows simultaneous increase in innervation of its horizontal recti.

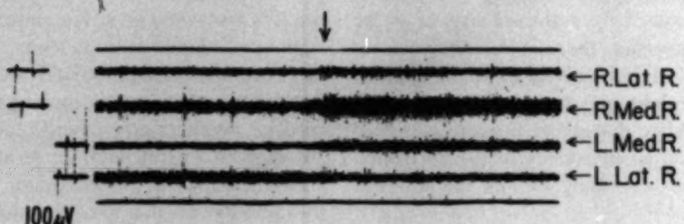
In Figure 6, we are recording both medial recti of another patient with approximately 15 degrees of intermittent exotropia. The bottom channel is the integrator display* of the activity of the first channel, the right medial rectus. The arrow indicates the point of uncovering the divergent left eye as the right eye continues to steadily fixate a light at near. As the left eye now converges to fuse, the stationary fixating right eye shows simultaneous increase in activity of its medial rectus, both on the direct trace and the integrator trace.

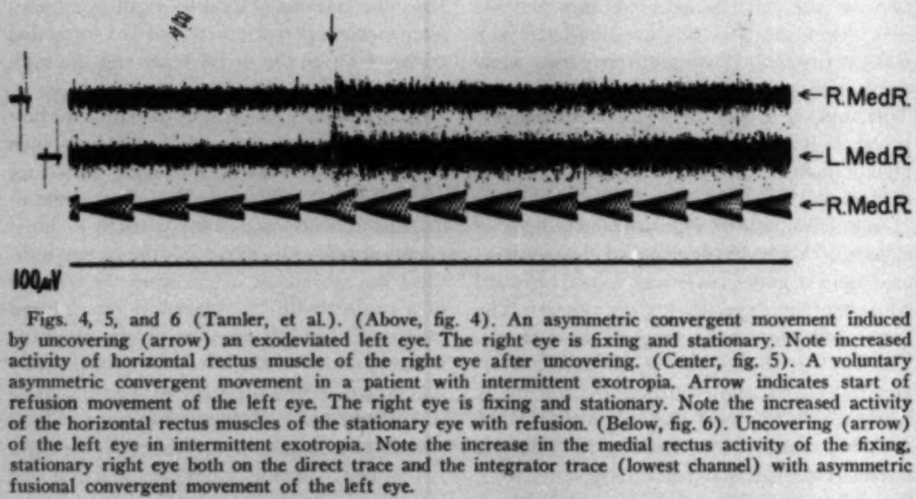
If a patient with an exo deviation is binocularly fixing at near, and one eye is then covered so that fusion is broken, the fixing stationary eye will then often show a simultaneous *decrease* in activity of both its horizontal muscles. In Figure 7, the arrow indicates the movement of covering the left eye of a subject with 15 degrees of intermittent exotropia. The left eye then diverges and the horizontal recti of the left eye are reciprocally innervated. When the left eye diverges, there is a simultaneous decrease in activity of both horizontal recti of the stationary fixing right eye. The following example, utilizing the integrator, shows this change more clearly.

In Figure 8, there is a decrease in activity of the medial rectus of the stationary fixing

* The integrator is an electronic device for taking the integral (sum) of the electromyographic potentials with respect to time. The vertical height of the step is proportional to the average value of the EMG electric potential without regard to polarity for the time interval between steps.

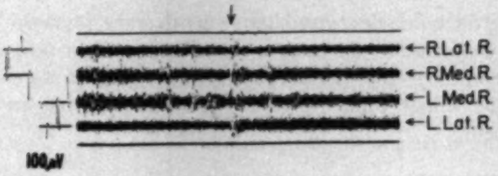


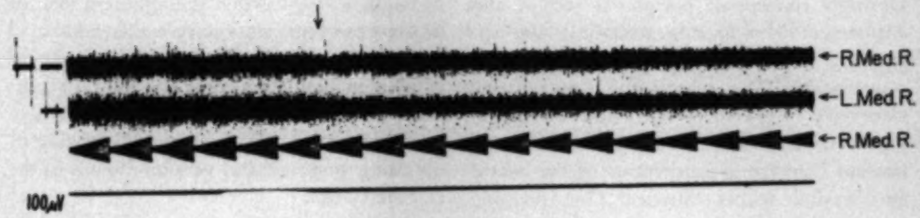


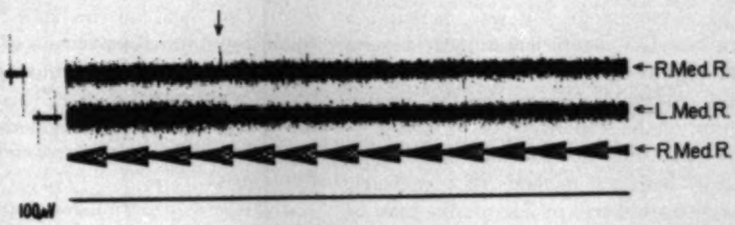


Figs. 4, 5, and 6 (Tamler, et al.). (Above, fig. 4). An asymmetric convergent movement induced by uncovering (arrow) an exodeviated left eye. The right eye is fixing and stationary. Note increased activity of horizontal rectus muscle of the right eye after uncovering. (Center, fig. 5). A voluntary asymmetric convergent movement in a patient with intermittent exotropia. Arrow indicates start of refusion movement of the left eye. The right eye is fixing and stationary. Note the increased activity of the horizontal rectus muscles of the stationary eye with refusion. (Below, fig. 6). Uncovering (arrow) of the left eye in intermittent exotropia. Note the increase in the medial rectus activity of the fixing, stationary right eye both on the direct trace and the integrator trace (lowest channel) with asymmetric fusional convergent movement of the left eye.

right eye as shown by both the direct trace and integrator trace, as the left eye of a 15-degree intermittent exotropia is covered (arrow). This simultaneous decrease in horizontal recti activity of the stationary eye with break in fusion is not found as consistently or seen as readily as the simultaneous increase in horizontal recti activity with refusion, even in the same patient. In Figure 9, for example, in the same patient as Figure 8, the left eye diverges after being covered (arrow). There is no obvious change in the trace of the right medial rectus. Yet in the same subject, a fusional convergent movement of the left







Figs. 7, 8, and 9 (Tamler, et al.). (Above, fig. 7). Covering (arrow) of the left eye in a subject with intermittent exotropia. The right eye fixing throughout. Note the decrease in activity of the horizontal rectus muscles of the right eye when fusion is broken. (Center, fig. 8). Covering (arrow) of the left eye in another subject with intermittent exotropia. The lowest channel is an integrator display of activity of the right medial rectus. Note the decreased activity of the medial rectus of the fixing right eye both on the direct trace and the integrator trace when fusion is broken. (Below, fig. 9). Same as in (center) Figure 8. No significant change in right medial rectus as fusion is broken.

eye never failed to give an observable increase in electric activity of the right medial rectus. The explanation for this apparent difference in refusion as compared to break in fusion is not clear to us at present.

The quick movement made by the "stationary" eye at the start of rapid asymmetric convergence, noted by Hering,[4] Westheimer and Mitchell,[2] and Alpern and Ellen,[3] can be seen sometimes by electromyography. Not all subjects show it for, as Hering[4] noted, it appears that trained observers show it less frequently than naive subjects.

In Figure 10, when the divergent left eye was uncovered (arrow), the fixing right eye was observed to make a quick conjugate movement to the right before returning to its previous fixing position. The trace of the right medial rectus shows a momentary inhi-

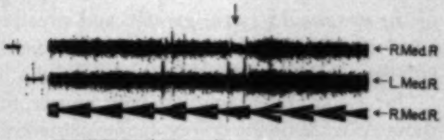


Fig. 10 (Tamler, et al.). When the left eye is uncovered (arrow), a rapid momentary conjugate movement of the right eye to the right was observed, followed by return of the right eye to its previous fixing position. Note the momentary inhibition of the right medial rectus, corresponding to this initial dextroversion.

bition accompanying the quick dextroversion movement which is confirmed by the integrator trace. This represents the peripheral manifestation of the initial rapid version innervation component.

Discussion

Our electromyographic data support Hering's concept of peripheral receipt and adjustment of opposing stimuli to the apparently stationary eye during asymmetric convergence. The reason the eye remains stationary is due to cocontraction of opposing horizontal recti, that is, there is a simultaneous increase in innervation of the lateral and medial rectus muscles. One possible reason why other investigators did not find this by electromyography was, perhaps, a failure to induce a sufficient angular amount of convergence in the moving eye in order to register observable changes on the electromyogram of the stationary eye.

It is interesting that Hering's law is supported by both test methods of asymmetric convergence utilized in his study, that of smooth, binocular convergence along the axis of one eye, and that of uncovering one eye to force a fusional convergent movement while the other eye continues to fixate.

It is of further interest that in breaking fusion we often find simultaneous decrease in innervation of the horizontal recti of the stationary eye. The necessity for such a decrease of innervation is easy to explain. If it did not occur, then continued refusion movements with repeated covering and uncovering of an eye would cause greater and greater build-up of electrical activity in the horizontal muscles of the stationary eye ad infinitum.

Our electromyograms not only appear to show evidence of the quiver of the stationary eye at onset of rapid asymmetric convergence, but, following this, reveal further peripheral changes in innervation despite the fact that the same eye subsequently remains stationary. This demonstrates that one may have a change in the electromyogram without necessarily having an associated eye movement.

Summary

1. Our electromyograms of asymmetric convergence reveal that the apparent lack of movement of the stationary eye is associated with a simultaneous increase of electrical activity in both its medial and lateral rectus muscles. This agrees with Hering's theoretical prediction of a peripheral balancing of opposing vergence and version stimuli to the stationary eye.

2. Our electromyograms of reciprocally acting horizontal muscles show evidence of a small, rapid initial movement of the stationary eye in asymmetric convergence which occurs with some subjects. This agrees with the work of other investigators who found a quick movement by nonelectromyographic techniques.

3. There is a peripheral manifestation of vergence and version in the horizontal recti of the stationary fixing eye as evidenced by two stimulus methods: (a) smooth binocular convergence along the axis of one eye; (b) the refusion movement which occurs when uncovering a previously covered abducted eye of an intermittent exotrope.

4. The evidence presented in this paper supports Hering's law and differs from other electromyographic investigators, both in interpretation of Hering's law and supporting electromyographic data.

Stanford University
School of Medicine (15).

Acknowledgment

We are indebted to Mr. William Houweling and Niles Roth, B.A., M.O., for technical assistance, and to Mr. Wilmer Renner for photographic assistance.

References

1. Breinin, G. M.: The nature of vergence revealed by electromyography. Arch. Ophth., **54**:407 (Sept.) 1955.

2. Westheimer, G., and Mitchell, A. M.: Eye movement responses to convergence stimuli. Arch. Ophth., **55**:848, 1956.

3. Alpern, M., and Ellen, P.: A quantitative analysis of the horizontal movements of the eyes in the experiment of Johannes Mueller: I. Method and results. Am. J. Ophth., **42**:289 (Oct. Pt. II) 1956.

4. Hering, E.: Der Raumsinn und die Bewegungen des Auges, in Hermann's Handbuch der Physiologie, Dritter Band, Vierterteil, Zweiter Abschnitt, Elftes Capitel, Leipzig, 1879, p. 519. (Or Hering, E.: Spatial senses and movements of the eye. [Translation from the original German by Carl A. Radde] Am. Acad. Optom., Baltimore, 1942.)

5. Blodi, F. C., and Van Allen, M. W.: Electromyography of extraocular muscles in fusional movement: I. Electrical phenomena at the breakpoint of fusion. Am. J. Ophth., **44**:136 (Oct. Pt. II) 1957.

6. Breinin, G. M.: The nature of vergence revealed by electromyography: II. Accommodative and fusional vergence. Arch. Ophth., **58**:623 (Nov.) 1957.

7. Dodge, R.: Five types of eye movements in the horizontal meridian plane of the field of regard. Am. J. Physiol., **8**:307, 1902.

8. Marg, E., Jampolsky, A., and Tamler, E.: Elements of technique of multiple channel electromyography of extraocular muscles in human subjects. In press.

9. Hering, E.: Ueber Muskelgerausche des Auges in Sitzungberichte d. Wien. Akad. d. Wissensch; Math. Naturw. Classe, Wien, **79**:II-III:LXXIV Band Dritte Abtheilung, 1879, p. 137.

Discussion

Frederick C. Blodi (Iowa City, Iowa): This is an extremely interesting and provocative piece of work. It is interesting because it concerns itself with a problem of basic oculomotor physiology and it is provocative because its results seem to be diametrically opposed to the conclusions reached by all previous authors working on this subject.

Before offering my questions and criticism may I congratulate the authors on their excellent technical result. I am especially impressed by the simultaneous recording from four muscles. This is not an easy feat as anybody can testify who has tried to test more than two muscles at the same time.

The author's slide 2 is one of the crucial electromyograms. It is supposed to show cocontraction of the horizontal antagonists during asymmetric convergence. I have two technical questions on this chart: (1) Why this unusual difference in level at which the various muscles are recorded? This makes the interpretation somewhat difficult; and (2) why this time-lag of more than one second after the breakpoint of convergence before the lateral rectus picks up in electrical activity?

The principal question, however, is "Does this eye remain really stationary when these electrical changes occur?" From the records available it seems that these cocontractions occur only just the moment before the breakpoint. It is possible that at this time the stationary eye begins to show some small movements. These may account for some of the increments in the electrical activity of the muscles concerned. In addition some subjects, especially untrained ones, may experience a defense-mechanism when an object comes too close to their eyes. This might lead to cocontraction, but has nothing to do with asymmetric convergence.

In the instances of intermittent exotropia the increase in electrical activity, especially visible in the medial rectus of the fixing, presumably stationary, eye is a phenomenon connected more with the fusional movement (which may induce a convergent movement in the fixing eye) than with any sustained effort we would expect in asymmetric convergence. This fusionl vergence may be preceded by a lateroversion as seen in Figure 10. That this is more a fusional vergence movement than a true cocontraction is also seen in Figure 9 in which no change occurs in the fixing eye as the nontested eye deviates.

It all boils down to the fact that changes in the electric potentials of the stationary eye are caused by small movements of adjustment at or near the break of asymmetric convergence. These changes have nothing to do with the steady state which exists in the stationary eye during this vergence. We have found this steady state to exist through a wide angle of convergence up to the break of fusion.

The authors were most kind to send me the manuscript months ahead of the meeting so that Dr. Van Allen and I had a chance to do some additional experiments on this problem.

The fusional component of asymmetric convergence can be studied on the haploscope which has the advantage that no object comes so close to the eye that a defense mechanism would be elicited. Our results show quite convincingly that there is no increase in the electrical activity of the horizontal muscles of the stationary eye. There may be a slight increment just before or after the breakpoint reflecting a slight quiver of the eye. This fact has been mentioned by us before.

We have repeatedly done asymmetric convergence while approximating an object in the visual axis of the stationary eye. The results were identical with those obtained at the haploscope. The fact that the horizontal muscles of the stationary eye do not recruit during asymmetric convergence becomes especially obvious when the patient first makes a lateroversion out of the range of the tested muscle. If then an object is approached along the visual axis of the stationary eye, the electric activity of

the tested muscle is and remains small. Even a slight increment could easily be picked up here. But none occurred. This experiment can of course also be done while the patient looks into the range of the tested muscle. Here the baseline of electric activity is high to begin with and changes may be more difficult to detect. None could be observed.

It seems to me therefore that on the basis of available evidence we must favor the theory of a central adjustment in the innervation of opposing forces and accept the fact that this adjustment is more subtle and efficient than a simple tug-of-war between the horizontal recti of the stationary eye. Cocontraction of antagonists occurs only in unusual or pathologic circumstances and the changes which have been shown to occur just before the break-point of fusion in asymmetric convergence in the authors' subjects is such a circumstance.

Dr. Edward Tamler (San Francisco): Dr. Blodi finds our paper provocative because our evidence is quite different from the previous electromyographic evidence on this subject. He feels we are probably wrong.

We would like to dissent very strongly from this feeling, for the following various reasons: In Dr. Blodi's own paper he shows one instance in asymmetric convergence where a lateral rectus muscle increases its activity by at least 20 percent in the stationary eye during the same type of asymmetric convergence.

Dr. Blodi says that perhaps this cocontraction that we see before the break of convergence is due to small movements of the eye. We pointed out that we cannot record small movements below eight or 10 degrees. The innervations we have recorded are rather large; they are equivalent to movements of at least 10 degrees, probably more.

The other point is that if we got a movement of the eye of any appreciable magnitude, we would see reciprocal innervation, not cocontraction of the horizontal recti muscles.

Then Dr. Blodi points out that perhaps these changes in the horizontal rectus muscles are part of a defense mechanism, a protective reaction of the patient to an object approaching close to the eye. We feel this is certainly not so, for the following reasons:

If you put an electrode into the auxiliary muscles, such as the superior rectus and inferior rectus and inferior oblique, and perform asymmetric convergence (this is convergence along the axis of the right eye), the right lateral rectus will show some increase in activity, but the other muscles, the inferior oblique or the vertical recti will show no change. If this were a defense mechanism one would expect every muscle to tighten up at the same time.

Furthermore, if one does asymmetric convergence with the moving eye occluded so that the only stimulus the stationary eye gets is an accommodative one, then there is no change in the horizontal recti muscles of the stationary eye. We have done this and find no change. Again, if this were a defense or protective mechanism one would expect some changes.

Furthermore, we had a couple of intermittently exotropic patients who, when fixing on a near target, showed unsteadiness of their fixation. One eye went in and out. The target remained at the same distance, but every time one eye changed fixation the other fixing, stationary eye had a simultaneous change in innervation of both horizontal recti. The target did not move, and yet these changes occurred. Again, we think this is evidence against a defense mechanism.

Furthermore, among the first few slides I showed you of smooth asymmetric convergence (figs. 2 and 3), after fusion was broken, the innervation of both horizontal recti decreased. The target was still coming in and yet the innervation was decreasing. Again, it is certainly evidence against this idea of a defense mechanism.

We have no doubt that cocontraction of the horizontal recti of the stationary eye does occur in asymmetric convergence. We have found it consistently and frequently enough to believe that our results are reliable.

Incidentally, the answer to Dr. Blodi's questions regarding slide 2 is that the difference in level of muscle activity and time lag is simply due to differences in muscle insertions. This matter is taken up in detail in our paper on normal variations and artefacts in electromyography, soon to be published.

I might mention also that the haploscope only goes in to 30 degrees and is probably not an adequate stimulus for these tests. We are grateful to Dr. Blodi for discussing our paper and for raising these interesting points. Dr. Alpern's comments are also appreciated.

ELECTROMYOGRAPHIC PATTERN OF SACCADIC EYE MOVEMENTS*

JAMES E. MILLER, M.D.
Saint Louis, Missouri

Horizontal eye movements have been shown to consist of two forms of motion: The very quick motion or saccadic movement and the slow or tonic change in position.[1] A saccadic movement is seen with conjugate fixational shift, while the tonic phase is present in vergence movements.

The pattern of movements has been recorded by photographing the corneal reflections or amplifying the relative positivity of the cornea as it moves between two fixed skin electrodes. These studies have demonstrated that saccadic movements are characterized by a simultaneous onset in both eyes with a rapid acceleration to a maximum velocity.[2] The ultimate velocity is proportional to the extent of movement. A 20 degree eye movement requires 70 milliseconds for completion.

In contrast vergence movements have a very slow acceleration. Consequently, activity persists for a much longer interval so that a six-degree convergence movement requires 800 msec. for completion.[3]

The marked difference between the two types of movement has been attributed to a dual innervation to the ocular muscles. The presence of large myelinated and small nonmyelinated efferent fibers supplying the muscle fibers has been advanced as support for this hypothesis.[4] The large fibers were considered to be the final common pathway for saccadic movement and the fine fibers the route for vergence movement.

It would seem that electromyography presents an opportunity to demonstrate the innervational aspect of saccadic eye movements. The changes that occur to produce motion are well illustrated. In addition innervational alterations which occur in the absence of movement are also shown.

I. METHOD

Recordings were made from eight normal subjects whose ages ranged from 21 to 62 years. Four muscles were explored in each experiment and the usual method involved obtaining tracings from pairs of antagonists. Adequate records were obtained from all muscles with the exception of the superior oblique.

The individual's head was placed in a headrest and a four-mm. light was viewed through a semitransparent mirror; the fixation distance was 18 feet straight ahead. At erratic intervals a second light was projected onto the mirror so that fixation of the second light required a conjugate movement of a known number of degrees. The movements were in steps of 2.5 up to 40 degrees.

For convergence movement the second light was placed in front of the distance fixation point so that a convergence of a given number of degrees was obtained. Recordings of 2.5-degree intervals up to 30 degrees of convergence were made.

The electrodes were of two types. The first type consisted of 30-gauge tubing through which was threaded an enameled stainless steel wire 75 μ in diameter. The wire was cemented in the center of the beveled needle opening. Connecting to the preamplifier were 40-gauge Litz wires, which are extremely flexible. The general myographic pattern was obtained with this electrode.

The second type of electrode was constructed of two enameled stainless wires 25 μ in diameter. The exposed ends were separated 50 μ from each other and cemented in the center of the bevel. The 30-gauge needle

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* From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University School of Medicine.

This investigation was supported in part by a research grant, B-1349, from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

was grounded and the recording made from the two connecting 40-gauge Litz wires. Single motor units could be isolated and followed through a range of motion by the second electrode.

The preamplifier was a dual channel Process and Instrument Company low level AC amplifier with a gain of 20,000. The amplified signal was fed to an Ampex FR-1100 FM tape recorder with a recording speed of 60 inches per second. This record could then be replayed at will and the oscilloscopic presentation photographed at various film speeds. In addition the playback speed of the tape recorder could be reduced eight times so that a time base expansion of eight fold was obtained. This permitted a greater familiarity with each recording since every phenomena could be photographed at various film speeds.

II. Results

Figure 1 illustrates a five-degree adduction saccadic movement. The stimulus appears as the interrupted spike in the beginning of the lateral rectus tracing. After a latent period of 0.28 seconds, there is an abrupt onset of fast firing motor units in the medial rectus which persist for 0.06 seconds. Concomitant with the burst of activity in the medial rectus, there is inhibition of the lateral rectus. After this activity stops a new pattern is established for the two muscles which continues for the remainder of the illustration.

In Figure 2, a range of motion from five

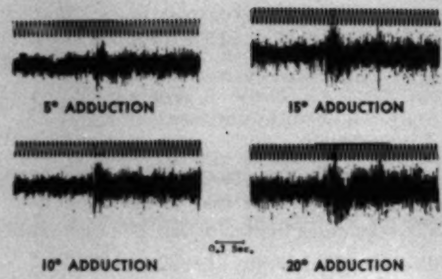

Fig. 2 (Miller). A range of saccadic motion from five to 20 degrees in a medial rectus.

to twenty degrees' adduction in a medial rectus is shown. With increasing motion the initial burst becomes prolonged and the ultimate pattern of fixation more active. The duration of the initial activity varied from 0.03 sec. for 2.5 degrees to 0.15 sec. for 40 degrees.

In the tracing of 15 and 20 degrees a second saccade is noted about 0.15 sec. following the initial activity. This is a characteristic of larger movements (15 degrees and above) and is thought to represent a second effort to overcome a lag in fixation. On several occasions these afterbursts have occurred in a serial fashion three or four times with a time separation of 0.15 to 0.2 sec.

This pattern is noted for all conjugate saccadic movement whether it be elevation, depression, or return to primary position from a secondary one. Figure 3 is illustrative of this, and in addition demonstrates the motor

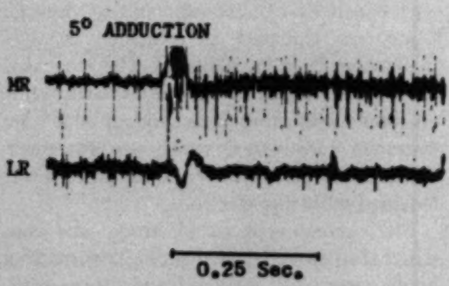

Fig. 1 (Miller). Tracing obtained from medial and lateral rectus during a five-degree saccadic movement.

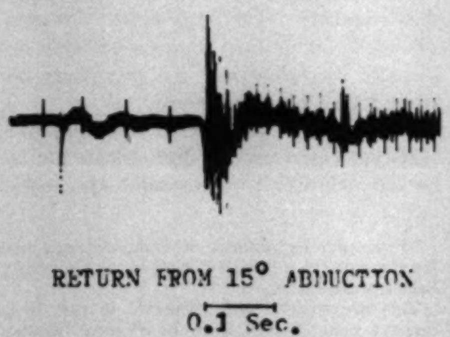

Fig. 3 (Miller). Pattern for conjugate saccadic movement.

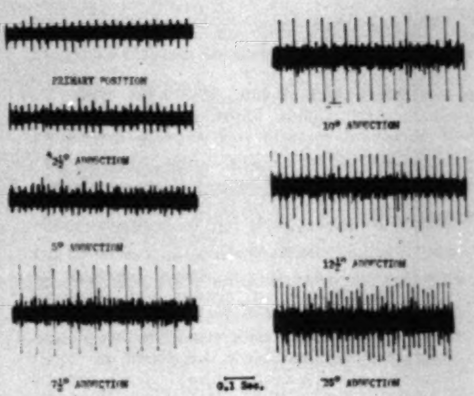

Fig. 4 (Miller). Motor unit patterns in a medial rectus one second after stimuli.

unit activity. The initial burst is immediately followed by an orderly series of steadily firing motor units. Upon this uniform pattern is superimposed the afterburst of a fixational lag.

Examination of the tracings taken one second after the stimulus (fig. 4) shows the orderly progression of firing when fixation is established. The rate of firing of a motor unit is related to the degree of contraction. With greater contraction, additional motor units are brought into play and these in turn increase their firing frequency according to the degree of fixation away from primary position. The converse is also seen with diminishing contraction.

Upon comparison of convergence (fig. 5) with adduction several differences are noted: (1) The onset of activity may be preceded by a short pause in firing; (2) the onset of convergence is more gradual and tends to rise to an innervational peak followed by a slow decline to the final innervational pattern; this decline may persist for over a second in convergence movement exceeding 15 degrees; (3) no afterbursts are seen during convergence.

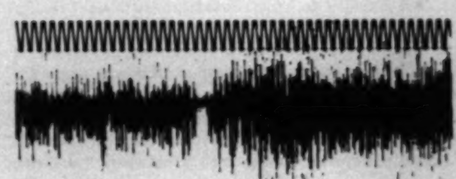

Fig. 5 (Miller). Fifteen-degree convergence pattern from a medial rectus.

III. Discussion

The innervational pattern of saccadic eye movement is in good agreement with the eye movements previously noted by other authors. The rising velocity that was noted by Westheimer would seem to be produced by the initial burst. As greater movement is called for, the beginning activity is prolonged. The duration of this saccade is also grossly equal to the time interval of the movement.

Another feature is the orderliness of the firing pattern once the initiating burst is completed. Motor units do not fire in a random fashion, but at a fixed rate. This firing rate is dependent on the angular displacement away from primary position. Fixation which requires additional contraction of a muscle mobilizes more motor units but these also fire at an orderly rate.

Saccadic movements are not characterized by "checking" action of the antagonist of the muscle that initiates the movement. When adduction is produced by a sudden innervation of the medial rectus, the lateral rectus is inhibited. As soon as the regular firing pattern resumes in the medial rectus, an orderly rate appears in the lateral. For large movements the initial burst is usually inadequate and one or more secondary saccades are necessary to bring the eye into position.

IV. Summary

1. A saccadic eye movement is induced by a sudden burst of motor unit activity which is immediately followed by an orderly firing pattern.

2. The duration of the initial burst is proportional to the extent of movement.

3. Large movements are usually inadequate and are corrected by a second saccadic burst.

4. A definite innervational difference is found between vergence and conjugate movements.

640 South Kingshighway (10).

References

1. Dodge, R.: Five types of eye movements in the horizontal meridian plane of the field of regard. Am. J. Physiol., **8**:307-329, 1902.

2. Westheimer, G.: The mechanism of saccadic eye movements. Arch. Ophth., **52**:710-718, 1954.

3. ———: Eye movement responses to convergence stimuli. Arch. Ophth., **55**:848-856, 1956.

4. Alpern, M., and Wolter, J. R.: The relation of horizontal saccadic and vergence movements. Arch. Ophth., **56**:685-690, 1956.

Discussion

Frederick C. Blodi (Iowa City): The essayist has presented electromyograms of unusual physiologic importance. The question is whether we can differentiate the basic mechanism underlying vergence and rapid version.

There is some histologic and neurologic evidence that such a differentiation can be made. We know that there are two types of fibers in the extraocular muscles. The thin ones in the periphery differ from those in the central part of the muscle. It is conceivable that these two types of muscle fibers are associated with two different types of muscle activity. The neurologic evidence for such a differentiation has been cited by the author.

The observed electromyographic evidence for this is quite interesting. The author has succeeded in differentiating two patterns of electric potentials, one for the rapid lateral gaze of the saccadic type and one for the vergence component of an eye movement. This observation may be more conspicuous because of the use of a bipolar needle. However, the phenomenon can also be observed when using the coaxial needle. I received the author's manuscript early enough to turn my attention to this point and I can only corroborate his findings.

Most interesting would be a more extensive study on the difference in the electric pattern between a saccadic movement and vergence. I have not seen a pause of silence preceding the firing, but I agree with the statement that afterbursts are not seen in convergence. The other statements on convergence were unfortunately not illustrated. This is certainly a most interesting approach and I hope that Dr. Miller will be able to continue his work in this direction.

Dr. Mathew Alpern (Ann Arbor, Michigan): In my opinion there is no conflict in the electromyographic evidence for the characteristics of the movements in asymmetric convergence. The difference, I believe, is in the kind of experiment that is being done.

If one shifts suddenly from A to B along the line of sight of one eye, I am quite sure that the changes in the muscles that were described in the first paper* can be clearly demonstrated. On the other hand, if one continues to fix an object as it is brought in along the line of sight of one eye, then I am quite sure the attempt to demonstrate these effects will lead to failure.

The reason is that a pursuit movement is a much more complex electrophysiologic process. Björk showed very neatly that a pursuit movement involved cocontraction of agonist and antagonist (Acta Ophth., **33**:437-454, 1955). Under these conditions it is difficult to understand how any electromyographic evidence could be found to verify Hering's law if the fixated object is slowly brought in along the line of sight of the eye being tested.

Dr. Henry Knoll (Los Angeles): I would like to comment on the second paper. Perhaps Dr. Marg or Dr. Jampolsky should be here. It is rather interesting, if one looks at some of the data available, that in these saccadic moments the eye is capable of angular velocities up to 600 to 800 degrees per second. In other types of fusional movements and the slow, drifting movements described by Ditchburn and Ginsbourg and others, the velocities are about 60 degrees per second. In the fast portion of the flicks (as Ditchburn calls them) the velocities are about 600 degrees per second.

As the author pointed out, Alpern has attempted to explain these differences on the basis of difference of innervation, and the records shown today indicate some of these differences as far as the muscular activity is concerned.

However, I think it is very important that we also look at the antagonistic muscles to see what they are doing. I believe Dr. Marg and Dr. Jampolsky have shown that in the slower movements the antagonist is being inhibited in a perfectly reciprocal manner. In fast movements the antagonist virtually drops out completely. It simply stops acting and permits the protagonist to act most effectively. It seems to be a most reasonable and efficient way of getting the eye up to speed as fast as possible.

I think before we can hope to find in this type of record support for Alpern's suggestion we will have to look carefully at what the other muscles are doing.

Dr. James E. Miller (closing): I would like to thank Dr. Blodi for his kind comments. We do plan to continue with this work.

We actually do have records of the lateral rectus. For simplicity's sake only that muscle was demonstrated which tends to produce the movement. I would like to point out that no claim was made for pathways in this work—only innervational patterns. We do not offer this as a support of dual innervation of the ocular muscles, only that an innervational pattern difference exists between these two types of movements.

* Tamler, et al., this publication, p. 174.

STUDIES ON THE IMMUNOLOGIC ORGAN SPECIFICITY OF OCULAR LENS*

Seymour P. Halbert, M.D., and Patricia L. FitzGerald, M.A.
New York

Uhlenhuth first showed that injection of lens substances from one species into another resulted in the production of precipitating anti-lens antibodies.[1] He also demonstrated that such antilens sera showed wide cross reactions with lenses obtained from a number of different animals in the vertebrate scale. Such extensive immunologic similarity has been termed organ-specificity. These studies were confirmed by several other investigators (for example,[2-4]).

In addition, Hektoen and Schulhof[4] examined the chemical fractions of bovine lens then known and concluded, on the basis of their results, that the organ specificity of the lens was due to immunologic similarities of the alpha and beta crystallines. These investigators also studied the cross reactions of the antilens sera among a number of vertebrate lenses including several species of fish. They found that the cross reactions of mammalian lens antisera with fish lenses were rather weak, and that the reverse was generally true.

Wollman, et al.,[5] extended and confirmed this type of observation in a short report, and found that rabbit antisera against mammalian lens (bovine, or horse) cross reacted with bird lenses, and with the lens of a cartilagenous fish (ray). They reported that antisera against octopus lens, however, failed to show any cross reactions with mammalian lens, while the reverse was similarly true.

All of the studies indicated above utilized simple precipitin tests in capillary or small tubes in a liquid medium. In recent years new and powerful sensitive immunologic methods have been developed which enable the estimation of the number and specificities of immunologic components in complex reacting systems. One of the most useful of these is the agar precipitin technique,[6-8] especially that developed by Ouchterlony.[9]

A report by Rao[10] utilized one of these methods in the examination of the number of immunologic components detected with rabbit antisera to bovine lens. He found six antigens in this system, two of which cross reacted with vitreous components. François, et al.,[11] studied the reaction of similar anti-bovine lens sera using the method of immunoelectrophoresis, a procedure which combines electrophoresis in agar gels followed by agar precipitin analysis. These investigators detected eight antigens in this way using bovine lens for the test. When they used horse or human lens, however, they could detect only one cross reacting antigen with this method while two cross reactions were noted with pig lens and three with sheep lens.

In previous reports, it has been demonstrated[12,13] that rabbits can be immunized with whole rabbit lens homogenate in Freund's adjuvant so that high titers of precipitating *homologous* antisera were produced. It was observed with such homologous lens antisera that at least five antigen-antibody systems were present when agar precipitin tests were performed with adult rabbit lens homogenates. No reactions were seen with these antisera and other rabbit organs. The homologous lens antisera were also tested for their cross reactivity with lens homogenates from other vertebrate species. Using the most potent antisera, only one cross reacting component was noted with the sea water fish (menhaden), two were found with fowl lens, four with frog lens homogenates, and four or five noted with guinea pig, rat, monkey, and human lenses.

* From the Departments of Ophthalmology and Microbiology, Columbia University, College of Physicians and Surgeons, and the Institute of Ophthalmology, Presbyterian Hospital, New York. This investigation was supported by a grant (B-1010 C) from the Division of Research Grants of the National Institutes of Health, Public Health Service.

It was the purpose of this present report to extend these observations with the use of heterologous lens antisera prepared in the rabbit.

Materials and Methods

Chinchilla rabbits (five to seven lb.) were used throughout the immunization. Lenses were obtained from the various species by careful dissection, homogenized with teflon glass homogenizer (A. H. Thomas Company, Philadelphia) with saline to give a final suspension of 100 mg. wet weight per ml. These were stored in aliquots in the deep freeze, samples being thawed as needed. The human cataractous lenses were obtained from the patients undergoing surgery at the Ophthalmological Institute. A single pool was made from several hundred such lenses for this study.

The squid (Loligo pealii) and the marine bony fish, menhaden (Brevoortia tyrannus), were obtained from a Long Island fishery and frozen within a short time following the catch. They were stored and shipped in the frozen state until the lenses could be dissected free. The frog lenses (Rana pipiens) were obtained from freshly killed animals.*

The lens homogenates were suspended in Freund's adjuvant,[12,14] and a total of 1.0 ml. of the adjuvant mixture was injected intradermally into six widely separate sites on each rabbit. With the doses of acid-fast organisms used, necrotizing ulcerations of the injection sites were the rule with repeated immunizations. The dose per rabbit for each immunization was 50 mg. of wet weight of lens. The quantity of insoluble fractions varied with the different species, but these were all included in the material injected.

Several courses of immunizations were made into the rabbits with sample bleedings obtained 10 days following the last injection of any series. Precipitating antibodies appeared after two doses, and the number and intensity of precipitin bands increased with subsequent injections. The sera used here consisted of samples obtained from rabbits that were given three to seven doses of antigen. The agar precipitin technique was the modification of the Ouchterlony method as previously described.[12]

Absorptions of the sera with the various antigens were carried out in liquid medium by adding the absorbing antigen and allowing precipitation to occur overnight in the refrigerator. After removal of the precipitates by centrifugation, more absorbing antigen was added and the process repeated until no further visible precipitation was obtained immediately.

Results

The type of results that were obtained with these sera are demonstrated in the following photographs. The antisera to human cataract lens (fig. 1) was tested against the other

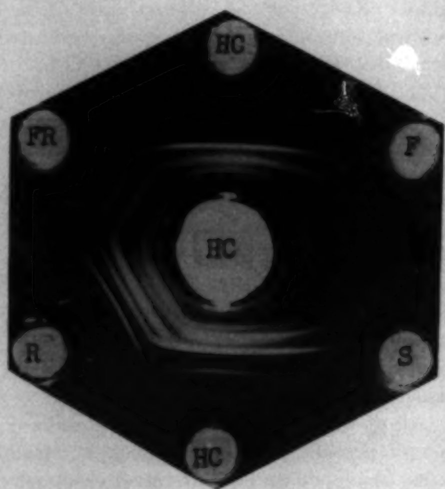

Fig. 1 (Halbert and FitzGerald). Agar precipitin test of antihuman lens antiserums against lenses of various species. Central well: HC = rabbit antiserum against human cataractous lens pool. Peripheral wells: F = lens of marine fish, menhaden; FR = lens of frog; R = lens of rabbit; S = lens of squid; HC = pooled human cataractous lens. All lenses were homogenized in 0.9-percent NaCl at 100 mg. wet weight/ml. Three days' development at 4°C.

* We are deeply indebted to Dr. L. G. Barth for his generous helpfulness in supplying the frog eyes.

antigens as noted, and it may be seen that seven components were observed when the human lens pool was used as test antigen. At least five cross reacted with rabbit lens, three with frog lens, and two with fish lens. No reaction at all was seen with squid lens.

In Figure 2 are shown the results found with the frog lens antisera. As may be noted, at least five components were observed with the immunizing frog lens homogenates, while four of these cross reacted with rabbit lens and at least three showed cross reactions with human cataract and fish lens homogenates. Again, the squid lens showed no cross reactions whatsoever.

Similarly, the fish lens antisera (shown in figs. 3 and 4) revealed at least four components, three of which cross reacted with frog lens, while two and possibly three reacted with human cataract and rabbit lens homogenates. Once again, the squid lens showed no precipitation at all with this antiserum.

The photographs demonstrate clearly one of the principal difficulties with these precipitin techniques, that is, the tendency of many of the bands to spread and overlap with its neighbor. In Figure 4 is shown the same plate as seen in Figure 3, but the former was photographed after five days of development compared to three days for the latter. The spreading of the bands seen with human cataract and rabbit lens is clearly apparent, with a tendency for striations to appear in these now wide and fuzzy precipitates.[15-17] Whether these represent artefacts or other systems requires further observations, but the band seen early in development almost certainly represent distinct antigen-antibody reactions. As also may be noted, a new component was visualized after five days of development that was not seen at three (see arrow, fig. 4).

In Figure 5 are shown the results of a test carried out with squid lens antisera. It may be noted that the squid lens homogenate showed at least four components, one being extremely dense, which implies relatively high concentrations of both antigen and anti-

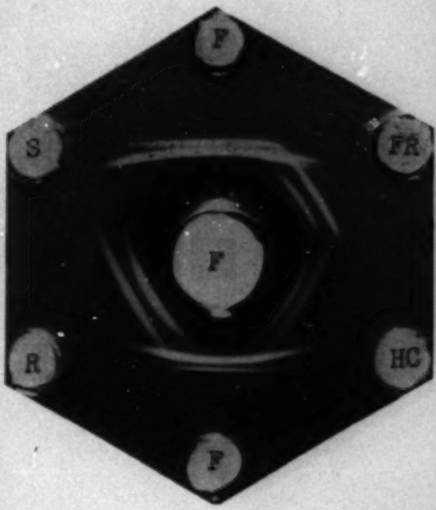

Fig. 3 (Halbert and FitzGerald). Agar precipitin test of antifish lens antiserum against lenses of various species. Central well: F = rabbit antiserum against fish lens. Peripheral antigen wells same as Figure 1. Developed at 4°C. for three days.

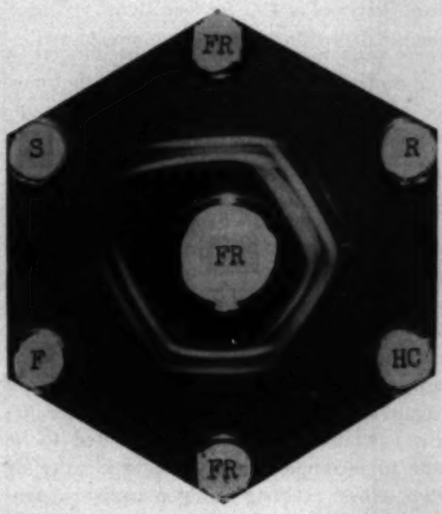

Fig. 2 (Halbert and FitzGerald). Agar precipitin test of antifrog lens antiserum against lenses of various species. Central well: FR = rabbit antiserum against frog lens. Peripheral well designations same as in Figure 1. Development three days at 4°C.

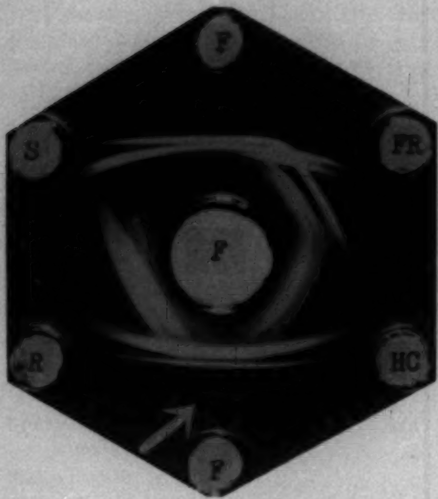

Fig. 4 (Halbert and FitzGerald). Same plate as in Figure 3 after five days' development at 4°C.

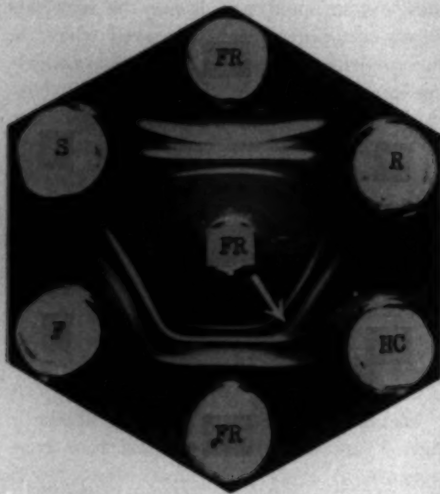

Fig. 6 (Halbert and FitzGerald). Precipitin tests of various lens antisera against frog lens homogenates. Central well: FR = frog lens homogenate, 100 mg. wet weight/ml. Peripheral wells: FR = rabbit antiserum vs frog lens; S = rabbit antiserum vs squid lens; F = rabbit antiserum vs fish lens; HC = rabbit antiserum vs human cataractous lens; R = rabbit antiserum against adult rabbit lens. Developed at 4°C. for four days.

body. The fish, frog, rabbit, and human cataract lens homogenates showed no reaction whatsoever with the squid lens antisera. If

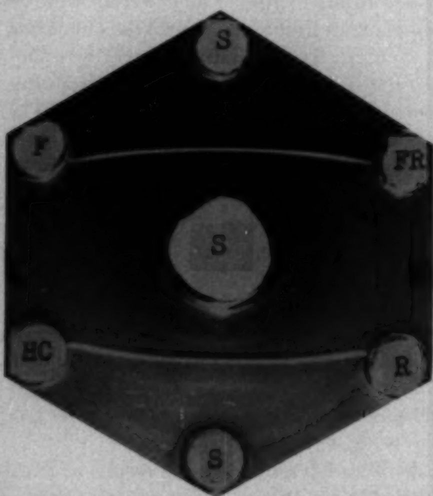

Fig. 5 (Halbert and FitzGerald). Agar precipitin test of antisquid lens antiserum against lenses of various species. Control well: S = rabbit antiserum against squid lens. Peripheral antigen wells, same as Figure 1. Developed at 4°C. for five days.

these tests were set up with the antigen in the central well, the type of results that may be seen are shown in Figure 6. In this instance, the homologous rabbit lens antiserum was rather weak and failed to show precipitin bands with the frog lens at this time of development, but it may be seen that the expected cross reactions were observed with the other systems. Figure 6 is shown principally because in the cross reactions between the middle component of the human cataract lens antiserum and the frog lens antiserum, the reaction of identity is strongly suggestive of the spur formation (see arrow) which has been demonstrated to be due to incomplete immunologic identity of two cross reacting antigen-antibody systems.[9, 18, 19]

In Figure 7 is shown the cross reaction between human cataract lens antiserum and a homogenate of bovine lens in which the latter was serially diluted. It may be pointed

out that different bands tend to have different dilution end points, which is a potent argument in support of their distinctness immunologically. There is a strong indication that at least six antigen-antibody systems were involved in this cross reaction, as best seen in the antigen well containing two mg. per ml. This evidence is supported by preliminary findings with antigen separations of bovine lens obtained by continuous flow electrophoresis.[23]

In Table 1 is shown the number of bands observed with the antisera and lens antigens used here. These figures represent the results of numerous assays, and undoubtedly reveal the minimum number of cross reacting components.

A series of tests were next carried out in which cross absorptions of the various antisera with each of the lens antigens were performed, and the resulting absorbed sera were tested for their content of agar precipitating antigens as described. In Table 2 is shown the rough estimation of the amounts of precipitates seen in each of the absorbing mixtures.

The agar precipitin test results with human cataract lens are shown in Figure 8. It can be seen that absorption of this serum with human cataractous lens, as expected, removed all of the precipitating antibody, while absorption of this serum with rabbit lens appeared to remove all but three of the

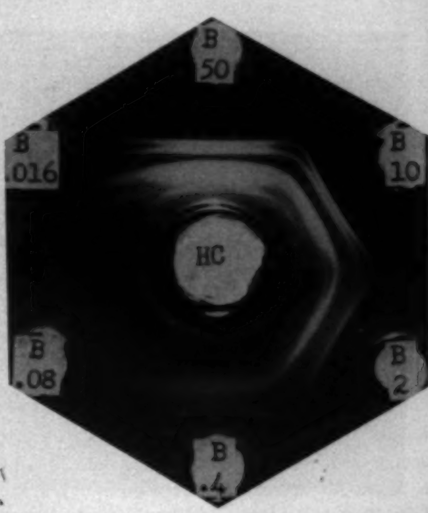

Fig. 7 (Halbert and FitzGerald). Serial dilution assay of bovine lens in its cross reaction with rabbit antiserum against human cataractous lens. Central well: HC = antiserum against human cataractous lens. Peripheral wells: Bovine lens homogenate. Figures refer to concentration mg. wet weight/ml.

antibodies involved. Absorption of the human cataract serum with frog lens clearly removed the heavy band component closest to the antigen well. This antigen, it may be seen, was present there (well HC/FR) in antigen excess and reacted with the non-absorbed antibody present in the well containing the human cataract lens antisera treated with fish

TABLE 1

LENS CROSS-REACTIONS WITH ANTILENS SERA

(Numbers of Bands)

Antiserum†	Antigen*				
	Rabbit	Frog	Human Cataract	Menhaden (fish)	Squid
Rabbit (homologous)	5	4	4	1	0
Frog	4	6	3	3	0
Cataract (human)	5	4	7	2	0
Menhaden (fish)	3	3	3	4	0
Squid	0	0	0	0	5

* All antigen lens homogenates at 100 mg. WW/ml.
† Prepared in rabbit.

TABLE 2

LENS CROSS ABSORPTIONS

Antiserum to Lens of	Absorbed with Lens of				
	Rabbit	Frog	Human (cataract)	Menhaden (fish)	Squid
Rabbit (homologous)	++*	±	+	±	0
Frog	++	+++	++	++	0
Human (cataract)	+++	++	++++	+	0
Menhaden (fish)	+	++	+	+++	0
Squid	0	0	0	0	++++

* Relative amounts of precipitate.

lens (HC/F). It may be noted that this antiserum absorbed with the squid lens seemed to have no detectable effect on the reaction with the cataractous lens.

In Figure 9 is shown the results of similar tests in which a frog lens antiserum was absorbed with the other lens antigens. Absorption with the immunizing frog lens was incomplete and the two components were still detectable between the frog lens antigen and the antiserum absorbed with frog lens. It also may be noted that absorption with rabbit lens failed to remove completely at least four antibodies against frog lens components. The fact that this well (FR/R) acted as antigen source and produced at least three bands with the frog lens antibodies present in well (FR), also suggests the possibility that the immunologic similarity between the frog and

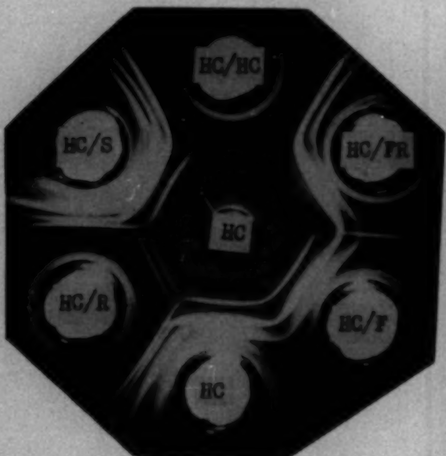

Fig. 8 (Halbert and FitzGerald). Absorption of antiserum against human cataractous lens with lenses of other species. Central well: Human cataractous lens homogenate. Peripheral wells: Rabbit antiserum against human cataractous lens. Absorbed with various lens homogenates; for example, HC/F = absorbed with fish lens; HC/S = absorbed with squid lens, and so forth.

Fig. 9 (Halbert and FitzGerald). Absorption of antiserum against frog lens with lenses of other species. Central well: Frog lens homogenate. Peripheral wells: Rabbit antiserum against frog lens absorbed with various lens homogenates; for example, FR/F = absorbed with fish lens; FR/S = absorbed with squid lens, and so forth.

the rabbit lens antigens are incomplete or that much larger numbers of antigen-antibody systems are involved here than the minimum indicated above. Similar findings were observed in well (FR/HC) where the frog lens antiserum was absorbed with human cataract lens. Again, it may be noted that absorption with squid lens failed to affect the antibody content of the frog antisera.

Essentially similar types of results were obtained with the menhaden lens antiserum absorbed with lens homogenates, as shown in Figure 10. Lastly, it was found that squid lens antiserum reactions were not affected at all by absorption with the other vertebrate lens homogenates, as is shown in Figure 11. Here again, absorption with the immunizing squid lens failed to remove completely one of the antibody components.

The minimum number of lens components detected between each of the lenses used and its own antiserum is not completely certain. These heterologous immunizations (for example, human lens with rabbit) may result in the production of nonlens antibodies and

Fig. 11 (Halbert and FitzGerald). Absorption of antiserum against squid lens with lenses of other species. Central well: Squid lens homogenate. Peripheral well: Rabbit antiserum against squid lens absorbed with various lens homogenates; for examples, S/F = absorbed with fish lens; S/S = absorbed with squid lens.

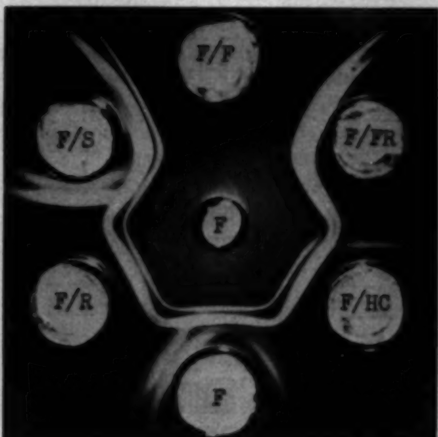

Fig. 10 (Halbert and FitzGerald). Absorption of antiserum against fish lens with lenses of other species. Central well: Fish lens homogenate. Peripheral wells: Rabbit antiserum against fish lens absorbed with various lens homogenates; for example F/F = absorbed with fish lens; F/S = absorbed with squid lens, and so forth.

this has been shown to be the case here. Some of the heterologous immunizations have resulted in the formation of varying small amounts of antibodies which react with other organ homogenates of the species used (for example, heart, liver, serum, and so forth). However, in appropriate tests it was shown that most if not practically all of these organ precipitin bands do not show reactions of identity with any of the readily visible antilens bands. This implies the presence of nonlens antigens in the lens homogenates in quantities too small to be revealed in the agar precipitin tests, but adequate to stimulate antibodies to them. Similar observations have been found in the immunologic analysis of saliva.[20]

The cross reactions between the antisera and the cross tested lens homogenates are almost certainly due to lens components, however. Preliminary results obtained in current tests with organ absorbed antilens sera also support the estimate of the lens components stated above.

Discussion

These findings reveal that the organ specificity of ocular lens in vertebrates may be based on the antigenic similarity of at least as many as six lens components. The number of the cross reactions of antibodies against these components varied with the species, and in general the fish system showed the fewest cross-reactions with the other vertebrate lenses.

Because of the difficulties noted with overlapping of precipitin bands as they develop, it is not possible to state at present the precise number of similar components in the various species, but it appears certain that complex and varying patterns exist throughout the animal kingdom.

In addition, suggestive evidence of reactions of partial identity (spur formation) and the data obtained with absorption studies point to the real possibility that some of these cross reactions are only due to incomplete similarity of the antigens involved. More extensive data, and purification of the antigens will be necessary to prove this.

The complete dissimilarity of all of the components of the squid lens and the vertebrate lenses studied confirmed in a conclusive way the observations made by Wollman with octopus lens. It is thus clear that cephalopod lenses do not contain any fractions which are similar immunologically to those seen in vertebrate eyes. On morphologic grounds, the development of the cephalopod eye is thought to have evolved along a different evolutionary path than the vertebrate eye.[21] The general structure of the eyes in the two animal classes is similar, and this has often been cited as an example of "convergent evolution."

The data presented here strongly support in a biochemical sense this concept that the evolution of these structures did indeed take place along different and distinct paths. It is of interest that the visual pigment chromophore group of the squid eye is apparently similar to that of the vertebrate eye.[22] This suggests perhaps that in the evolutionary development of the eye, the photosensitive receptors appeared at a very early period, and remained largely unchanged while the superstructure evolved along the two paths mentioned. It is planned to carry out immunologic studies of lens or lens analogues from species throughout the animal kingdom in order to obtain more extensive information of this nature.

Summary

1. Antisera were prepared in rabbits against lenses from several vertebrate species (human, fish, and frog), and from the invertebrate, squid. The immunologic cross-reactions among vertebrate lenses were found to be due to antigenic similarity of varying numbers of the components detected, in some cases being at least as high as six.

2. The invertebrate squid lens was shown to be completely distinct immunologically from the vertebrate lenses tested. These immunologic findings support the evidence suggesting that the cephalopod eye and the vertebrate eye evolved along distinct lines.

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References

1. Uhlenhuth, P. T.: Festchr. zum 60 Geburstag. Robert Koch, Jean, Fischer, 1903, pp. 49-74.
2. Woods, A. C., and Burky, E. L.: J.A.M.A., **89**:102-110, 1927.
3. von Dold, H., Flössner, D., and Kutscher, F.: Ztschr. f. Immunforschg., **46**:50-58, 1926.
4. Hektoen, L., and Schulhof, K.: J. Infect. Dis., **34**:433-439, 1924.
5. Wollman, E., Gonzalez, P., and Ducrest, P.: C. Rend. Soc. Biol., **127**:668-670, 1938.
6. Oudin, J.: Methods Md. Res., **5**:335-378, 1952.
7. Elek, S. D.: J. Clin. Path., **2**:250-258, 1949.
8. Oakley, C. L., and Fulthorpe, A. J.: J. Path. & Bact., **65**:49-60, 1953.
9. Ouchterlony, O.: Acta Path. & Microbiol. Scand., **26**:507-515, 1949.
10. Rao, S. S., Kulkarni, M. E., Cooper, S. N., and Radhakrishnan, M. R.: Brit. J. Ophth., **39**:163-169, 1955.
11. Francois, J., Rabaey, M., Wieme, R. J., and Kaminski, M.: Am. J. Ophth., **42**:577-584, 1956.

12. Halbert, S. P., Locatcher-Khorazo, D., Swick, L., Witmer, R., Seegal, B., and FitzGerald, P.: I. J. Exper. Med., **105**:439-452, 1957.
13. ———: II. J. Exper. Med., **105**:453-462, 1957.
14. Freund, J., and Bonanto, M. V.: J. Immunol., **48**:325-334, 1944.
15. Grabar, P.: Ann. N.Y. Acad. Sci., **69**:591-607, 1957.
16. Wilson, M. W., and Pringle, B. H.: J. Immunol., **73**:232-243, 1954.
17. Halbert, S. P.: J. Exper. Med. **108**:385-410, 1958.
18. Korngold, L.: J. Immunol., **77**:119-122, 1956.
19. Wilson, M. W., and Pringle, B. H.: J. Immunol., **77**:324-331, 1956.
20. Ellison, S. A., and Mishimo, P. A.: J. Dental Res., **38**:28, 1958.
21. Gregory, W. K.: Our Face from Fish to Man. New York & London, Putnam, 1929.
22. Hubbard, R., and St. George R. C. C.: J. Gen. Physiol., **41**:501, 1958.
23. Halbert, S. P.: Unpublished observations.

Discussion

Phillips Thygeson (San Jose, California): This excellent study is the third of a series dealing with the organ specificity of the lens—a subject of great interest not only to ophthalmologic investigators but to immunologists, biochemists, and all clinicians concerned with the fascinating problems of auto-immunization and disease. The authors review the literature from the pioneer work of Uhlenhuth (1903) who first showed that injection of lens substances from one species into another resulted in the production of precipitating antilens antibodies, and that such antilens sera showed wide cross-reactions with lenses obtained from different animals in the vertebrate scale. They stress the studies of Hektoen and Schulhof (1924) who related organ specificity to the immunologic similarities of the alpha and beta crystallines, and the studies of Wollman and associates who extended these investigations and reported that antisera against octopus lens failed to show cross-reactions with mammalian lenses, and vice versa.

The development in recent years of more sensitive immunologic methods, especially the agar precipitin technique, has enabled investigators to estimate the number and specificities of the immunologic components in lens reactions. In their previous reports the present investigators were able to demonstrate that at least five antigen-antibody systems were present when agar precipitin tests were performed with adult rabbit lens homogenates, and that no cross-reactions were noted with such lens antisera and other rabbit organs. They were able to show that such antisera had only one cross-reacting component with lenses from the sea-water fish menhaden, two with fowl lens, four with frog lens, and four or five with guinea-pig, rat, monkey, and human lenses. Their present report extends in an important way these earlier investigations.

The studies today reported clearly indicate that organ specificity of the ocular lens in vertebrates is based on antigenic similarity of six or more lens components, and that cross-reactions of antibodies against these components definitely vary with the species. The finding that fish lenses showed the fewest cross-reactions with the other vertebrate lenses was not unexpected. In view of the varying patterns existing in the animal kingdom, I am wondering if present data would permit the identification of unknown lens material on the basis of characteristic patterns.

The authors have noted difficulties concerned with the overlapping of precipitin bands as they develop, and with reactions of partial identity (spur formation). It is to be hoped that further studies will overcome these difficulties so that the precise number of similar components in the various species can be determined. The results of further studies with organ-absorbed sera will be awaited with much interest.

The authors' demonstration of the complete dissimilarity of all of the components of the squid lens and the vertebrate lenses confirms conclusively the observations of Wollman and associates with the octopus lens. These immunologic findings certainly lend support to the theory that the cephalopod eye and the vertebrate eye evolved along different lines.

The authors' plan to extend their observations by carrying out immunologic studies of lens or lens analogues from species throughout the animal kingdom is an exciting one and their results will be eagerly awaited. May I congratulate them on an exceedingly well planned and executed series of studies in a most important and interesting field?

Dr. E. C. Ferguson (Iowa City, Iowa): I would like to point out the practical significance of this excellent experiment.

I think all of us in ophthalmology realize that the present skin test with bovine and human lens material for phacogenic uveitis is very impractical. It is quite evident that there are several substances producing many different responses in the human eye. I refer to one paper by Dr. Irvine in which he reported many different responses from the cellular and pathologic standpoints.

It is hoped that eventually we will have several substances which may be used for skin tests in order to determine what substance is causing the phacogenic uveitis.

Dr. Seymour P. Halbert (closing): First, I would like to thank Dr. Thygeson for his very kind

remarks. With regard to the separation of these various antigens, we are currently planning and have started experiments in which we hope to isolate each of these components as we detect them immunologically. It is our hope that this will not only separate the antigens involved but perhaps will give us a clearer understanding of the complexity of proteins in the lens of the eye.

I do not know what will be found with regard to hypersensitivity responses of these various fractions, but this will also have to await purification of the components themselves.

RADIOELECTROPHORETIC PATTERNS OF AQUEOUS AND PLASMA*

After intravenous injection of I^{131}-labeled insulin into rabbits

Kenneth M. Giles,† B.S. and John E. Harris, M.D.
Portland, Oregon

An appreciable body of evidence indicates that the movement of glucose into the lens is metabolically mediated. This conclusion is based largely upon the following observations. First, the accumulation of glucose by the lens is reduced by certain metabolic poisons.[1,2] Second, when the integrity of the lens surface is disrupted as by decapsulation a rather marked decrease in glucose utilization is found.[3,4] Third, glucose moves across the lens capsule more rapidly than another metabolite (acetate) of smaller size.[5]

Since glucose moves into the lens by some process other than simple diffusion, an influence of insulin on such movement might be anticipated if the lens behaves like other tissues. The effect of insulin on glucose utilization and accumulation by the isolated lens has been extensively studied. The results have varied somewhat depending upon whether the lens was intact or decapsulated. Ross observed that the addition of insulin markedly increased glucose utilization of the decapsulated lens,[6] an observation which we have confirmed qualitatively but not quantitatively.[7,8] Thus, although we used conditions similar to those of Ross, we found a considerably smaller increase in glucose utilization, the uptake approaching that noted when insulin was added to a lens with a nicked capsule.[1] On the other hand, insulin has no apparent effect on the movement of glucose across the intact capsule in vitro as measured both by analysis[1] and by the accumulation of radioactivity from C^{14} labeled glucose.[8] This phenomenon has been observed in diabetic preparations as well.

Farkas and Patterson, however, have recently reported in vivo studies showing increased glucose utilization by isolated lenses from either diabetic or normal rats pretreated with insulin. This increase was abolished by total evisceration and appeared to require the presence of an intact liver.[9] These results are contrary to those of the in vitro studies and indicate that insulin may have a direct effect on the intact lens in vivo.

This seeming discrepancy can best be resolved by determining whether insulin enters the aqueous humor in physiologically significant concentrations. The present study reports the results of attacking this problem by investigating the migration of I^{131}-labeled insulin into the anterior chamber of the rabbit.

I^{131} activity of aqueous and plasma after injection of I^{131}-labeled insulin

Fifty µc. per kg. body weight of I^{131}-labeled insulin as received from a commercial source* were injected intravenously into male albino rabbits. At various time intervals

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† Recipient of a Fight-for-Sight Summer Student Fellowship Award of the National Council to Combat Blindness, Inc., Summer, 1957.

* Abbott Laboratories.

thereafter, aqueous was drawn from the anterior chamber and a blood sample was taken by cardiac puncture. An equal volume of one-percent serum albumin was added to the aqueous and the proteins of both plasma and aqueous were precipitated with 10-percent trichloroacetic acid. The precipitate was washed three times with five-percent trichloroacetic acid, then dissolved in 30-percent KOH. The first wash was added to the supernatant and the activity of both the precipitate and supernatant solution was measured by means of a shielded Geiger-Mueller tube and scaling unit.

The trichloroacetic acid precipitable activity in the plasma was found to decrease rapidly after the injection of the labeled insulin (fig. 1). This curve presumably reflects the diffusion of insulin into its space of distribution (calculated to be 25.8 percent of body weight[10]) and a continual degradation of the insulin molecule itself. As a result of the latter process, there was a progressive decrease in precipitable activity and increase in activity of trichloroacetic acid soluble material. Activity of the latter reached a maximum after about one hour, then slowly decreased. These data conform essentially to those of Scott, et al.[10]

The trichloroacetic acid precipitable activity in the aqueous rose to a peak at about 45 minutes, then gradually dropped to a low level at four hours (fig. 2).

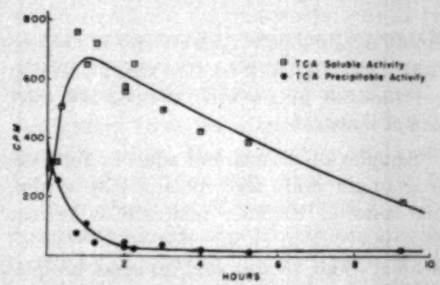

Fig. 1 (Giles and Harris). TCA precipitable and soluble activity in rabbit plasma following injection of I^{131}-labeled insulin. The activity was measured under conditions of different geometry from Figures 3 to 12 and the absolute values are not directly comparable.

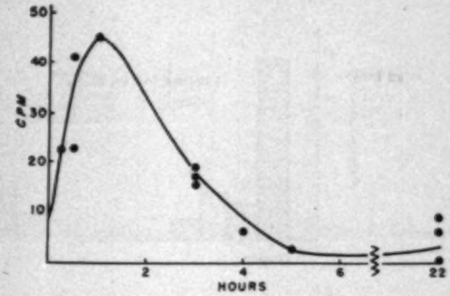

Fig. 2 (Giles and Harris). TCA precipitable activity of aqueous following intravenous administration of I^{131}-labeled insulin. The activity was measured under conditions of different geometry from Figures 3 to 12 and the absolute values are not directly comparable.

Although nearly 100 percent of the biologically active insulin is precipitated by trichloroacetic acid,[11] the converse, namely that all the activity in the coagulum represents insulin, cannot be assumed to be true. Impurities present in the insulin or other substances to which released I^{131} may become bound may also be found in the precipitate. To determine the true insulin therein a further step to distinguish between I^{131} attached to insulin and that attached to other trichloroacetic acid precipitable material is essential. For this purpose electrophoretic separation of the protein constituents on paper was employed.

Radioelectrophoretic patterns of I^{131}-labeled insulin

To determine the feasibility of identifying I^{131}-labeled insulin by means of paper electrophoresis, preliminary studies of the electrophoretic behavior of the anticipated amounts of labeled insulin under certain conditions were necessary. Small amounts of the labeled insulin were subjected to paper electrophoresis using the Beckman/Spinco Model R paper electrophoresis system, in barbital buffer, pH 8.6, ionic strength 0.075, at 2.5 ma. constant current for 16 hours. In certain instances carrier insulin or other pro-

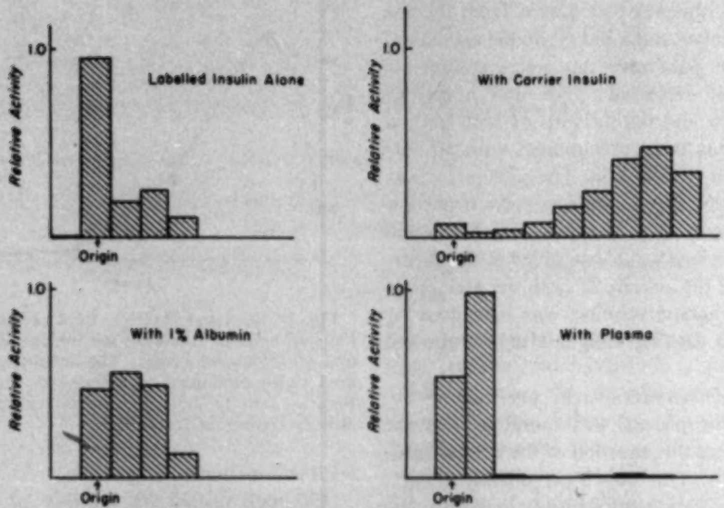

Fig. 3 (Giles and Harris). Radioelectrophoretograms of I^{131}-labeled insulin with and without carrier proteins.

teins were added. After electrophoresis the strip was stained, cut into sections one centimeter wide, and the activity of each section determined by counting under a shielded Geiger-Mueller tube. In general 10 such sections were assayed after each run. It had previously been determined that essentially all the activity on the strip was included in this area.

Labeled insulin remained within the boundaries of the strip which included the point of application and the next adjacent strip (fig. 3). When carrier insulin was added the I^{131} activity moved some seven cm. down the strip to a point which corresponded to that of the stainable band of insulin. These results are essentially the same as those reported by Berson, et al.[11] However, albumin and insulin migrate at approximately the same rate and separation of activity due to insulin and that due to albumin is not feasible by this technique.

When plasma or serum was added to the labeled insulin, the pattern of radioactivity was essentially that obtained with the I^{131} insulin alone, that is, the activity remained at the origin and the next adjacent strip. It appears, therefore, that the most exact separation of insulin activity is achieved by electrophoresis of the unaltered plasma samples or of aqueous to which plasma or serum albumin is added as carrier for the non-insulin proteins. Under these circumstances that activity remaining at the origin and on the adjacent strip may be presumed to be insulin activity.

Radioelectrophoretic patterns of plasma and aqueous after intravenous administration of labeled insulin to normal rabbits

Samples of plasma and aqueous for electrophoretic study were obtained by cardiac and anterior chamber puncture at various time intervals after injection of I^{131}-labeled insulin. Both plasma and aqueous samples were applied to strips of filter paper two millimeters in width cut from regular electrophoretic strips, air-dried, then placed at the origin of regular sized strips for the run. The

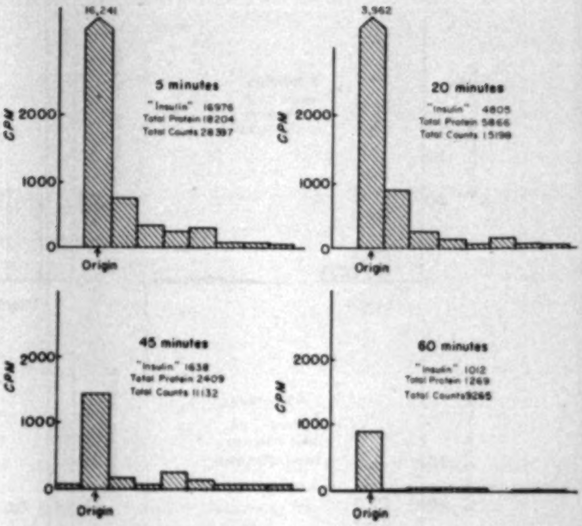

Fig. 4 (Giles and Harris). Representative radio electrophoretograms of normal rabbit plasma at various time intervals after intravenous administration of I^{131}-labeled insulin. "Insulin" activity = activity at the origin and the next adjacent section. Total protein = the sum of the individual bars. Total counts = total activity before electrophoresis.

volume of plasma used was six microliters. The aqueous volume was measured and varied from about 0.15 ml. to 0.25 ml. A six microliter sample of plasma drawn from the animal before the injection of insulin was also applied to the strip on which the aqueous was run. After electrophoresis the strips were stained, sectioned, and counted. Results are presented as counts per minute in 0.2 ml. of aqueous or plasma.

Radioelectrophoretograms of plasma drawn at various times after intravenous injection of labeled insulin are shown in Figure 4. No significant difference was seen in the amount of activity migrating with the plasma proteins at the various time intervals. However, the total activity within the first two centimeters from the origin showed a progressive decline. This most likely represents biologically active insulin. At least, it can be accepted that most of the adsorbed material is unaltered insulin since it comprises a major fraction of the insulin-I^{131} preparations which show no loss of biologic potency.[11]

Relatively similar patterns are seen in radioelectrophoretograms of aqueous taken at progressive time intervals after intravenous administration of labeled insulin (fig. 5). A small but significant amount of activity was found to remain at the origin. That this material is probably insulin is confirmed by the observation that the addition of carrier insulin results in migration of the radioactivity down the strip (fig. 6). As with the plasma samples there was no significant difference with time in the amount of activity migrating down the strip with the carrier plasma protein added to the aqueous sample.

The rate of degradation of insulin activity may be estimated by plotting the amount of activity remaining in the first two centimeters against time (fig. 7). As with the trichloroacetic acid precipitable activity a rapid decrease in activity in the plasma samples was observed. In the aqueous samples a rise in insulin activity to a peak level at 30 to 60 minutes was followed by a gradual decline at later time intervals. It should be noted that the activity in the aqueous attained only a small fraction of that found in the plasma. The decline in radioactivity in the aqueous may represent in small part an enzymatic degradation of insulin by the intraocular structures but is more likely due to the pas-

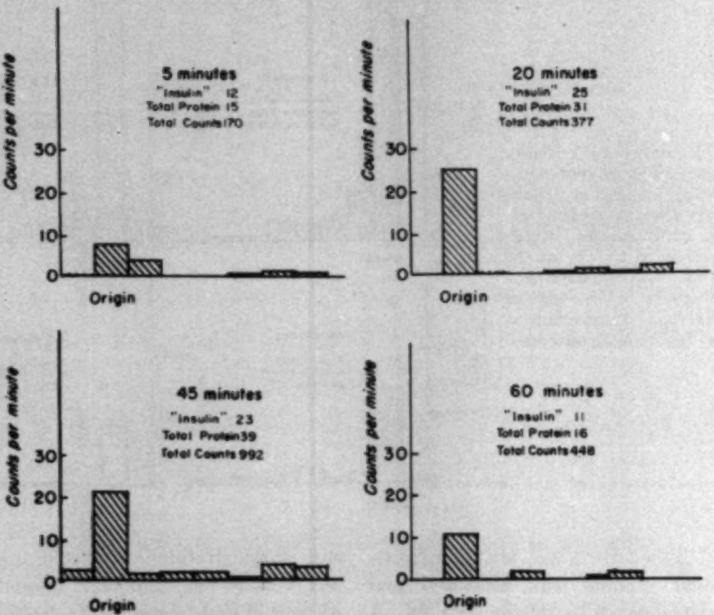

Fig. 5 (Giles and Harris). Representative radioelectrophoretograms of normal rabbit aqueous at various time intervals after the intravenous administration of I^{131}-labeled insulin. "Insulin" activity = activity at the origin and the next adjacent section. Total protein activity = the sum of the individual bars. Total counts = total activity present before electrophoresis.

sage of the labeled protein from the anterior chamber.

Effect of diabetes and exogenous glucosemia on the radioelectrophoretograms of aqueous and plasma

Diabetes was induced in rabbits by injecting 175 mg. of alloxan monohydrate per kg.

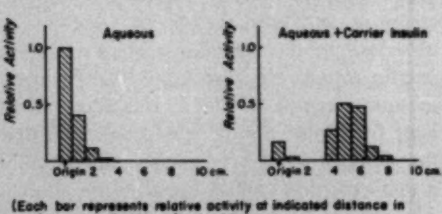

Fig. 6 (Giles and Harris). Representative radioelectrophoretograms of paired aqueous samples with and without added unlabeled carrier insulin. The samples were taken 30 minutes after the intravenous administration of I^{131}-labeled insulin.

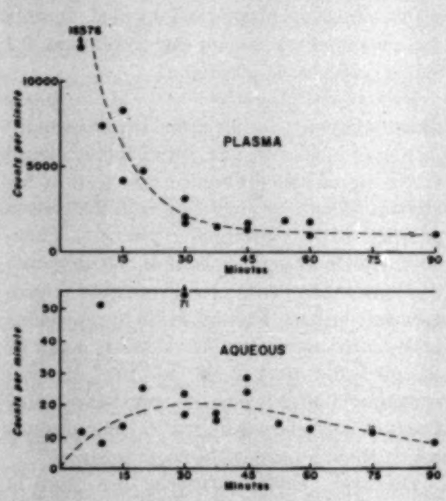

Fig. 7. (Giles and Harris). "Insulin" activity of plasma and aqueous in normal rabbits following intravenous administration of I^{131}-labeled insulin.

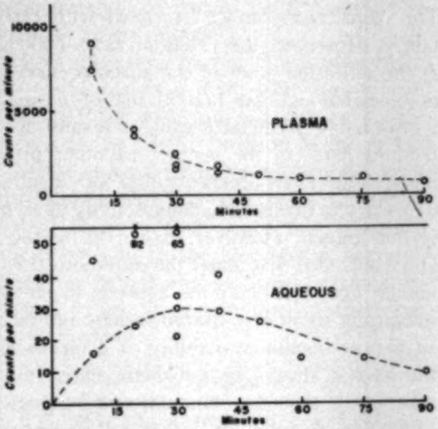

Fig. 8 (Giles and Harris). "Insulin" activity of plasma and aqueous in diabetic rabbits following intravenous administration of I^{131}-labeled insulin.

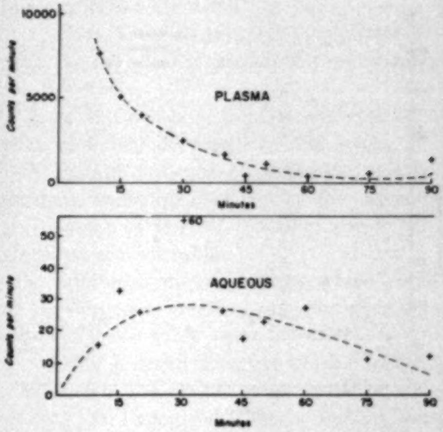

Fig. 9 (Giles and Harris). "Insulin" activity of plasma and aqueous in exogenous hyperglycemic rabbits following intravenous administration of I^{131}-labeled insulin.

body weight. The animals were maintained without insulin. Their blood sugars were at least 400 mg. percent at the time of the administration of the labeled insulin. Hyperglycemia was induced in normal rabbits by the combined intravenous and intraperitoneal administration of 50-percent glucose solution. Blood glucose levels were maintained above 400 mg. percent in these animals during the experimental procedure.

Following the injection of I^{131}-labeled insulin, the time course of the insulin activity of plasma and aqueous samples was essentially the same in diabetic rabbits (fig. 8) and in normal rabbits with induced hyperglycemia (fig. 9). These two experimental situations gave essentially the same pattern of activity as did the normal animals when the rate of insulin degradation in the plasma and the amount of activity accumulating in the aqueous were compared. When a composite curve of the three situations is made (together with data from a normal rabbit pretreated with 0.5 units of insulin per kg. body weight 15 minutes before the injection of the labeled insulin) the plasma values overlapped (fig. 10). A similar composite of aqueous insulin activity plotted against time showed a wider spread of the data but no outstanding difference between the groups (fig. 11).

It should be noted that radioelectrophoretograms of plasma samples from the various experimental situations showed essentially the same migratory pattern (fig. 12).

Discussion

The results indicate that some substance which is presumably labeled insulin enters

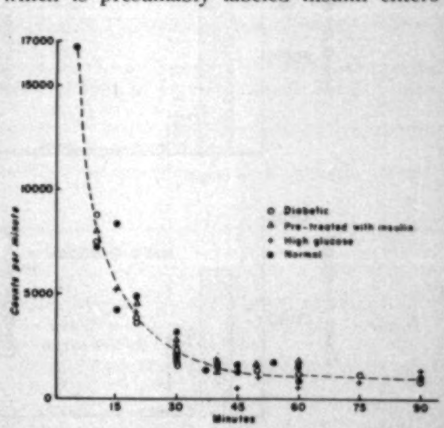

Fig. 10 (Giles and Harris). Plasma "insulin" activity after intravenous administration of I^{131}-labeled insulin.

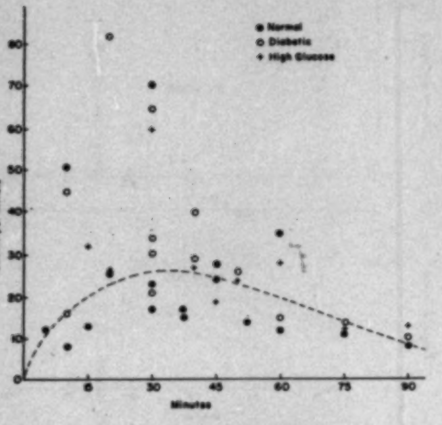

Fig. 11 (Giles and Harris). Aqueous "insulin" activity after intravenous administration of I^{131}-labeled insulin.

the anterior chamber after the intravenous injection of I^{131}-labeled insulin. However, the level of activity obtained in the aqueous after a single injection of the labeled insulin is only a small fraction of the level in the plasma even at the peak of aqueous activity. The exact ratio cannot be stated with certainty. However, the essential ratio (taken at the inflection point of the aqueous curve) is somewhat less than 1/100th that of plasma, a reasonably predictable value if insulin distributes itself in the manner of other proteins. The exact concentration of insulin necessary to maintain biologic activity in vivo is not known. However, it can be reasonably concluded that under the conditions used insulin does not enter the aqueous in physiologically significant concentrations and that so far as insulin availability is a measure, the lens is always in a diabetic state. It is also likely that the stimulation of glucose utilization observed by Farkas and Patterson to follow pretreatment with insulin did not result from the migration of insulin into the aqueous.[9] (The possibility that a partial degradation product of insulin, which retains biologic activity but is either devoid of I^{131} or is so small as to migrate off the paper, may be present in the aqueous but not detected by our method seems remote. Scott, et al., found that the biologic activity of the

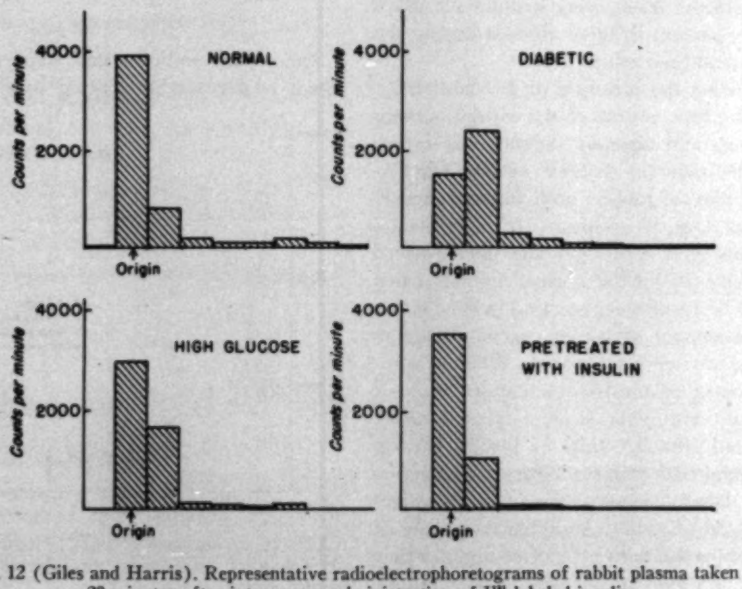

Fig. 12 (Giles and Harris). Representative radioelectrophoretograms of rabbit plasma taken 20 minutes after intravenous administration of I^{131}-labeled insulin.

plasma paralleled that of the I^{131} activity remaining at the origin.[10])

It is interesting to note that insulin enters the cerebrospinal fluid in insignificant amounts[12] as well and probably is not required for metabolic function of the central nervous system. One can reasonably speculate that insulin does not cross the blood-retinal barrier either, since this is similar physiologically to the blood-brain barrier.[13] That the retinopathy of diabetes seems unrelated to insulin requirement is, therefore, not surprising.

Summary

Following the intravenous administration of I^{131}-labeled insulin to normal, diabetic, and hyperglycemic normal rabbits the following observations were made:

1. The trichloroacetic acid precipitable activity in the plasma decreased rapidly after the injection.

2. In the aqueous, this activity rose to a peak and then decreased to low levels.

3. Similar patterns of radioelectrophoretograms were obtained from the aqueous and plasma of normal, diabetic, and hyperglycemic normal rabbits.

4. Similar levels of activity which could be considered as unaltered insulin were also obtained from these same animals.

5. Only a small fraction of the activity measured in the plasma which could be considered as biologically active insulin was found in the aqueous humor.

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References

1. Harris, J. E., Hauschildt, J. D., and Nordquist, L. T.: Transport of glucose across the lens surfaces. Am. J. Ophth., **39**:161 (Feb. Pt. II) 1955.
2. Mueller, H. K.: Ueber die Resorption von glucose und askorbensaüre in die Linse. Arch. f. Ophth., **140**:258, 1939.
3. Giles, K. M., and Harris, J. E.: Unpublished data.
4. Michail, D., and Vancea, P.: Nouvelles recherches sur le pouvoir glycolytique du cristallin. Compt. Rend. Soc. de Biol., **109**:1011, 1932.
5. Heinrichs, D. J., and Harris, J. E.: Lens metabolism with the reversible cation shift: IV. The ability of various metabolites to replace glucose. Am. J. Ophth., **42**:358 (Oct. Pt. II) 1956.
6. Ross, E. J.: Nature, **171**:125, 1953.
7. Giles, K. M., and Harris, J. E.: Unpublished data.
8. ———: Influence of insulin on the glucose transport across the lens surface of normal and diabetic animals. Am. J. Ophth., **43**:290 (Nov. Pt. II) 1957.
9. Farkas, T. G., and Patterson, J. W.: Insulin and the lens. Am. J. Ophth., **44**:341, 1957.
10. Scott, G. W., Prout, T. E., Weaver, J. A., and Asper, S. P.: A comparison of the behavior of insulin and insulin labeled I^{131} in serum. Diabetes, **7**:38, 1958.
11. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. S. and Newerly, K.: Insulin I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin treated subject. J. Clin. Invest., **35**:170, 1956.
12. Haugaard, N., Vaughan, M., Haugaard, E. S., and Stadie, W. C.: Studies of radioactive injected labelled insulin. J. Biol. Chem., **208**:549, 1954.
13. Palm, E.: On the occurrence in the retina of conditions corresponding to the blood-brain barrier. Acta Ophth., **25**:39, 1947.

Discussion

John W. Patterson (Vancouver, British Columbia): I would like to emphasize the importance of this very excellently presented paper. To do so I would like to make some general remarks related to the subject.

We know that patients and animals with insulin—deficiency diabetes, such as juvenile diabetes, diabetes that follows pancreatectomy, and alloxan diabetes, will develop cataracts if the condition is untreated. Cataracts can be prevented by injecting adequate amounts of insulin or by providing a diet that contains energy-yielding foods that are not dependent on insulin for utilization.

Since glucose under ordinary circumtances provides most of the energy for maintaining the transparency of the lens, it must be concluded that insulin is essential if the usual metabolic pathways of the lens are to remain active, but that it is possible to utilize alternative pathways that do not require insulin.

Further, we know that cataracts following insulin deficiency are not the result of high blood-sugar levels nor the secondary urinary changes that follow, because cataracts can be prevented with special diets in the presence of hyperglycemia. Therefore, we conclude that insulin must play a more direct role in lens metabolism.

It has been pointed out in the presentation that glucose utilization in isolated lens preparations is not stimulated by insulin. However, the injection of insulin in an animal some time prior to sacrifice will increase glucose utilization in the isolated lens. This difference in in vitro and in vivo results may be explained in one of three ways: First, insulin may require a co-factor before it acts in the lens.

Second, insulin may have to be altered or modifiel before it is active in the lens.

Third, insulin may be responsible for the formation of an unknown substance in some other organ such as the liver, which in turn is active in lens metabolism.

The work which has been presented is of great importance in this type of reasoning, to differentiate between the various possibilities. It has been shown that very little of the radioactive insulin enters the aqueous, and the feeling has been expressed that the assay represents the physiologically active form of insulin—in other words, that it is not broken down into some other molecule which would not be determined by this assay method.

Therefore, we must conclude that the first two of these three possibilities, namely, that a co-factor is required as well as insulin or that insulin in some modified form may be active, are untenable, and it leaves us with the third possibility.

We must conclude, therefore, that insulin acts in lens metabolism through some other organ.

Studies in our laboratory, which will be reported in detail at another time, suggest that this substance may be produced in the liver.

These conclusions as presented in this discussion are based on the premise that small amounts of insulin entering the aqueous are not physiologically significant. As the authors have pointed out, we do not know how many insulin molecules are required to produce a physiologic effect. Therefore, we must still maintain some reservation even though we accept the conclusion as being probably correct.

In closing I would like to ask the speaker one question. In their procedures for this assay, albumin or plasma was added prior to electrophoresis. I wonder if the speaker would be good enough to comment on the effect of various amounts of protein as related to their passage in the electrophoretic current.

Dr. Bernard Becker (St. Louis): Just a very brief comment so that the ciliary body is not entirely neglected.

There is evidence accumulated by Ross and others that insulin has an effect on transport mechanisms of the ciliary body. Therefore, there is still a fourth alternative explanation. Ones does not have to go as far away as the liver necessarily. One could have an effect of insulin on ciliary epithelium and on secretory processes, and thereby affect the lens metabolism.

Dr. Kenneth M. Giles (closing): First, I would like to thank Dr. Patterson for his fine discussion, and Dr. Becker for his comments on Ross' work.

Concerning the question about the different amounts of carrier unlabelled protein added to the insulin, we tried several different concentrations of plasma and albumin added to the labelled insulin. The amounts we added did not move the labelled insulin from the origin. However, small amounts of unlabelled insulin also will not move the labelled insulin from the origin, but larger amounts of unlabelled insulin do move the labelled insulin from the origin. When a similar amount of other unlabelled proteins is added to the solution, the activity does not migrate from the origin.

Basically, carrier insulin will move the labelled insulin from the origin in concentrations that carrier plasma does not move from the origin. These concentrations were used throughout the procedure.

11-CIS VITAMIN A IN THE PREVENTION OF RETINAL ROD DEGENERATION*

An animal study

Albert Chatzinoff, M.D.
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and

Nathan Millman, Ph.D., William Oroshnik, Ph.D., and Fred Rosen, Ph.D.
Raritan, New Jersey

Dr. George Wald[1] demonstrated three years ago that the all-trans form of vitamin A was unable to produce rhodopsin in vitro. He showed that the isomer, which he designated neo-B, produced rhodopsin. The precise structure of this isomer was unknown at that time.

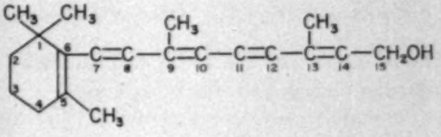

Fig. 1 (Chatzinoff, et al.). Vitamin A.

Figure 1 demonstrates the all-trans form of vitamin A. This structure is found in nature and in our vitamin capsules. In the normal individual an isomerization must take place somewhere in the body to produce the neo-b isomer.

Figure 2 demonstrates the then-known isomers of vitamin A. These isomers were the unhindered forms of vitamin A. At that time, the sterically hindered forms were believed to be impossible structures.

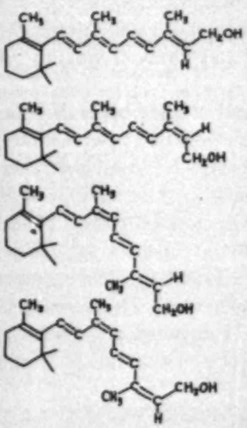

Fig. 2 (Chatzinoff, et al.). Biologic activities of the unhindered vitamin A isomers.

Figure 3 demonstrates the sterically hindered 11-cis vitamin A. The existence of this form was proven possible by one of us (W. O.) who demonstrated with spectroscopic proof that the neo-B isomer was in reality 11-cis vitamin A. At Dr. Wald's laboratory, it was this isomer that produced quantitative yields of rhodopsin in vitro.

Figure 4 demonstrates what we believe to be the in vivo rhodopsin cycle. In 1956, Dr. Ruth Hubbard[5] isolated a retinene isomerase, as is shown in this cycle. To date, no vitamin A isomerase has been isolated.

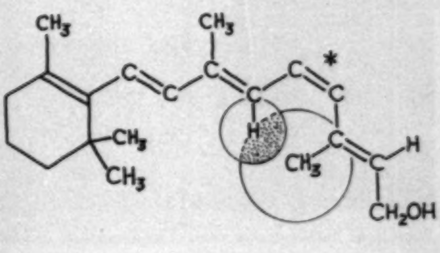

Fig. 3 (Chatzinoff, et al.). 11-cis (neo-b).

Dr. Norman Krinsky[2] added to our knowledge of the specificity of 11-cis vitamin A for the eye when he demonstrated that the only

* From the Ortho Research Foundation, Raritan, New Jersey, and the Mount Sinai Hospital, New York. Dr. Chatzinoff was Research Fellow of the National Council to Combat Blindness. Dr. Rosen is presently at Roswell Park, Buffalo, New York.

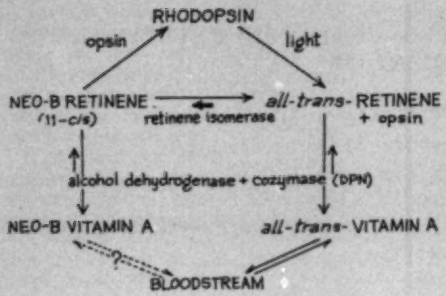

Fig. 4 (Chatzinoff, et al.) In vivo rhodopsin cycle.

tissue in the body containing this isomer was the pigment epithelium of the retina.

It was our concept that in view of the essentiality of the 11-cis isomer of vitamin A to produce rhodopsin, and the importance of the latter in the normal physiology of the retinal rods, it was reasonable to assume that 11-cis vitamin A was also essential for the normal metabolism and ultimate viability of the rods.

Early experimental studies by both Tansley[3] and Johnson[4] showed that newborn rats, deprived of all vitamin A from birth, developed somatic changes of vitamin-A deficiency (weight loss, xerophthalmia) and retinal changes marked initially by retinal rod degeneration followed by degeneration of other layers of the retina. It was our belief that if we could demonstrate that subminimal doses of 11-cis vitamin A would prevent retinal changes, but not somatic changes, we would have evidence, in the animal, for protection of the retinal rods, and perhaps other retinal structures by this isomer.

Newborn rats were given intramuscular injections of the vitamin from the time of birth. Twice minimal, minimal and sub-minimal doses of all-trans vitamin A and 11-cis vitamin A were administered during the period of time in which all other vitamin-A intake was eliminated from the diet of the animal.

Experimental results

Figure 5. These animals were placed on a completely vitamin-A free diet from the time of birth for a period of seven weeks. There

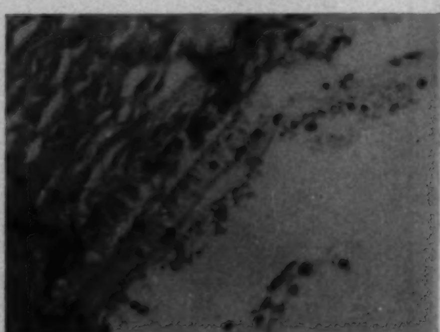

Fig. 5 (Chatzinoff, et al.). Degeneration of all retinal layers.

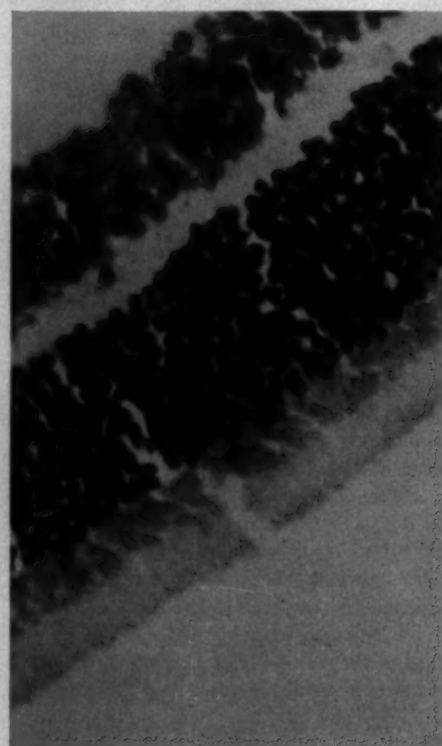

Fig. 6 (Chatzinoff, et al.). The retinal rod structure remained normal.

was marked weight loss, diarrhea, xerophthalmia, and some showed cataracts. The retina shows complete degeneration of all retinal layers.

Figure 6. These animals were given 15 units of all-trans vitamin A for four weeks. There were no somatic changes and the retinal rod structure remained normal.

Figure 7. These animals were given eight units of all-trans vitamin A for seven weeks. This being the basic minimal daily requirement, there still occurred in many cases (such as in figure 7) early fragmentation of the outer limb of the rod layer. These animals showed xerophthalmia.

Figure 8. These animals were given four units of all-trans vitamin A for six weeks. This was half the minimal daily requirement and the animals showed weight loss and xerophthalmia. The retina showed fragmentation and granulation of the rod layer and disruption of the external nuclear layer.

Figure 9. These animals were given 16

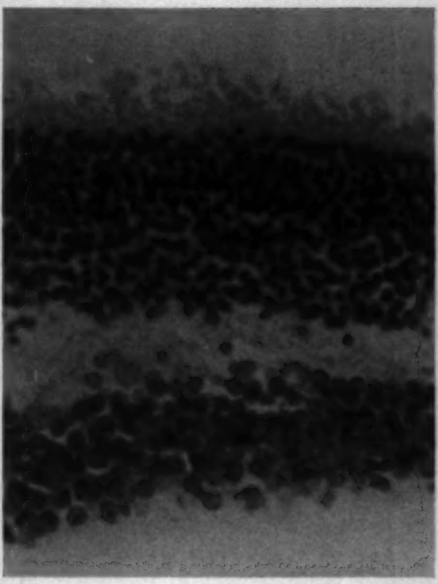

Fig. 8 (Chatzinoff, et al.). Fragmentation and granulation of the rod layer and disruption of the external nuclear layer.

Fig. 7 (Chatzinoff, et al.). Fragmentation of the outer limb of the rod layer.

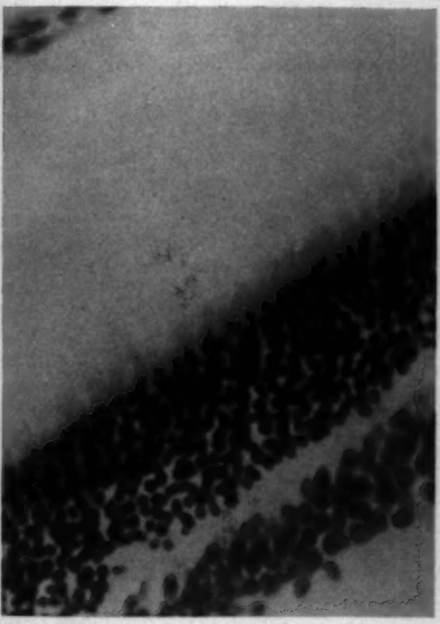

Fig. 9 (Chatzinoff, et al.). The retina was normal.

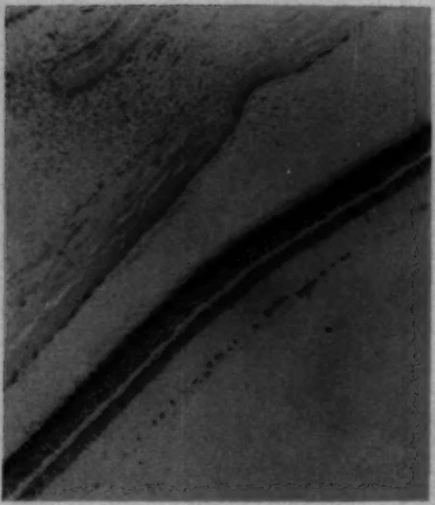

Fig. 10 (Chatzinoff, et al.). The retina was normal.

units of 11-cis vitamin A for eight weeks. They showed loss of hair over the entire body and retarded growth. The retina was normal.

Figure 10. These animals were given eight units of 11-cis vitamin A for eight weeks. They showed weight loss, but several showed

Fig. 12 (Chatzinoff, et al.). The retina was normal.

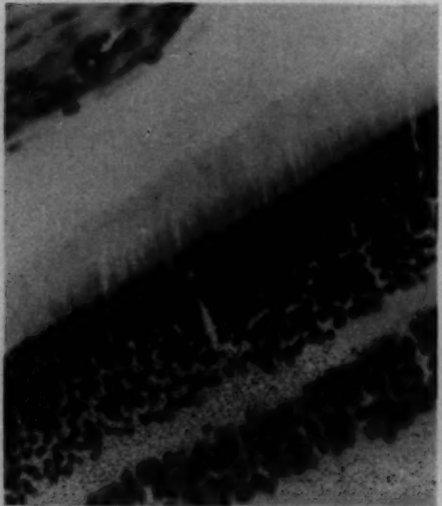

Fig. 11 (Chatzinoff, et al.). The retina was normal.

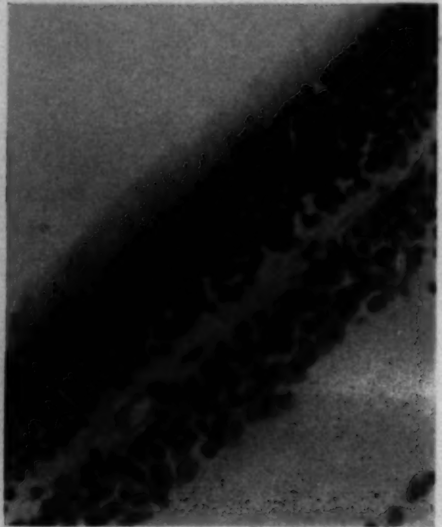

Fig. 13 (Chatzinoff, et al.). The retina was normal.

no somatic change in this group. The retina was normal.

Figure 11. These animals were given four units of 11-cis vitamin A for eight weeks. They showed diarrhea and weight loss. The retina was normal.

Figures 12 and 13. These animals were given two units of 11-cis vitamin A for eight weeks. They showed diarrhea and weight loss. The retina was normal.

Summary

This animal study showed:

1. Subminimal doses of 11-cis vitamin A will protect the retina of the newborn rat but did not prevent somatic changes of vitamin A deficiency.

2. Subminimal doses of all-trans vitamin A did not do so.

3. This study strongly suggests that 11-cis vitamin A is essential for the viability as well as the normal physiology of the retinal rods.

4. The normal individual must isomerize the all-trans vitamin A or all-trans retinene somewhere in the body to the 11-cis form. In retinitis pigmentosa, we postulate an hereditary or constitutional metabolic inability to make this isomerization. Clinical studies based upon this hypothesis are now being pursued.

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References

1. Wald, G.: Paper read before the Association for Research in Ophthalmology at A.M.A. Convention—June 1955.
2. Krinsky, N.: Personal communication to National Council to Combat Blindness.
3. Tansley, K.: Factors affecting the development and regeneration of visual purple in the Mammalian retina. Proc. Roy. Soc., London S.B., **114**:79-103 (Nov.) 1933.
4. Johnson, M. L.: The effect of vitamin A deficiency upon the retina of the rat. J. Exper. Zool. **81**:67-89 (June) 1939.
5. Hubbard, R.: J. Gen. Physiol., **39**:935-962. (July) 1956.

Discussion

Dr. Jerry H. Jacobson (New York): There is one further bit of information which has been forthcoming from Dr. Wald's laboratory that has a bearing upon the clinical complications that Dr. Chatzinoff presented, and that is that he has shown that if you take the lipid component of rod pigment and the lipid component of cone pigment and put them together in a beaker, and add an insufficient amount of 11-cis vitamin A to this combination in the beaker, the cone pigment is most active and picks up most of the vitamin A.

This could be an explanation of why the cone mechanism is preserved longer than the rod in diseased eyes, since the cones would be more active in picking up whatever is available and therefore would live longer. This would be an added confirmation of Dr. Chatzinoff's concept.

Dr. Albert M. Potts (Cleveland): I think one ought to emphasize again the difficulty of the work that was carried out by these experimenters. In a letter received from Dr. Millman who did the animal experimentation I was told that only two or three animals were usable in the experimental group. This was either because of succumbing to vitamin-A deficiency or because of fragmentation of the retina on sectioning in these deficient animals. So, perhaps, a larger number of animals would be desirable before drawing final conclusions. If the conclusions are correct, however, there are some fascinating implications that do result.

One of these is that the isomerase which is responsible for transforming all-trans-vitamin A to the 11-cis isomer must of necessity exist only in the retina and not elsewhere in the body, as was at one time thought; for, if this were not true, the small suboptimal dose of 11-cis compound given parenterally would be lost to the vitamin A-hungry body, and very little of it would get to the retina to exert the effects that were observed.

The second implication is that when retinene is bleached, and if we assume that not only is the all-trans form created by light but that the linkage between the lipoid and the protein is ruptured, then one should have equilibrium between the all-trans compound in eye and in circulation. Again this all-trans material, if it were allowed to escape from the retina, would be lost for any physiologic effect. Hence, one must also postulate that the 11-cis compound, even though it may be isomerized to all-trans retinene, never escapes from the retina. There is a fascinating possible explanation of why this is true.

It was mentioned by Dr. Wald some years ago that vitamin A, when it is found in the retina, is

not found as a free compound but esterified. There has never been an adequate explanation for the function of esterification in the visual cycle. This may actually be a vitamin-A conservation mechanism for the retina. If esterification occurs with a nondiffusible acid, then the little bit of 11-cis compound necessary for viability of the retina may, even when it is isomerized all-trans-A, still stay put in the retina and be available for reuse as 11-cis compound for the service of the retina alone and not for the deficient animal body.

DR. ALBERT CHATZINOFF (New Hyde Park, New York:

First, as I was trying to indicate in my studies, I would like to stress the fact that these animals were not easily preserved because of severe vitamin-A depletion. Although small numbers of animals were ultimately pathologically studied, the striking and uniform nature of the response to 11-cis vitamin A was particularly noteworthy and, I believe, quite significant. We are planning a repeat of this study.

We felt that this initial study was worthwhile presenting when we found that as little as two units of 11-cis vitamin A were sufficient to preserve a normal retina despite somatic changes in the animal. In addition, the facts that have emanated from Dr. Wald's laboratory have added tremendously to our knowledge concerning the part that the 11-cis-isomer plays in retinal physiology.

I hope that in the future, 11-cis vitamin A will prove to be an important weapon to the practicing ophthalmologist.

ELECTRIC ACTIVITY OF CELLS IN THE EYE OF LIMULUS*

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Bethesda, Maryland

The horseshoe crab (Limulus polyphemus) has two composite lateral eyes each containing several hundred ommatidia. Each ommatidium contains a variable number of retinula cells and one eccentric cell which are arranged as shown in Figure 1. The optic nerve includes large axons originating in eccentric cells and smaller axons presumed to originate in retinula cells. In the vicinity of the center of the ommatidium, the membranes of neighboring retinula cells form a structure called rhabdome. This structure has been recently examined by means of electron microscopy and is supposed to have the function of a photoreceptor (Miller, 1957).

Lateral eyes excised from the animal survive for a long time and produce impulses following illumination. The responses recorded from optic nerve bundles have been extensively studied, especially by Hartline and his co-workers (see, for instance, Hartline and Graham, 1932; Hartline, Wagner, and MacNichol, 1952).

Continuing work initiated by other authors (Hartline, Wagner, and MacNichol, 1952; MacNichol, 1956; Tomita, 1956), some features of the responses recorded by means of microelectrodes inserted in cells of eyes

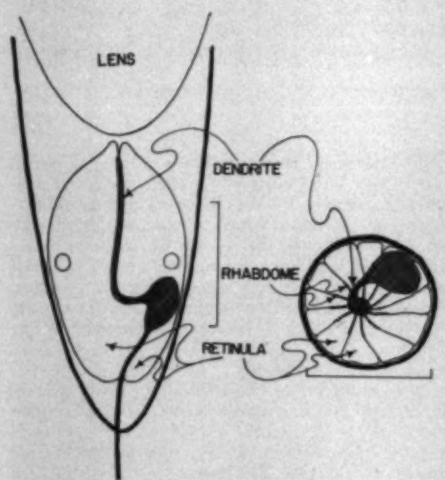

Fig. 1 (Fuortes). Diagram of the ommatidium. The longitudinal section at left shows the transparent light cone (lens) and, under it, large retinula cells with their nuclei and one eccentric cell (shaded) with its long "distal process" or "dendrite" and large axon. The cross section at right shows the radial arrangement of the retinula cells, the rhabdome, and the eccentric cell with its "dendrite" in the center of the ommatidium. The line is 100 μ.

* From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

of horseshoe crabs will be discussed in the present paper.

Method

The eye was dissected from the animal, cut in two along its longest diameter, and placed in artificial sea water. With the aid of a dissecting microscope individual ommatidia can be clearly seen in this preparation but the cells contained in them cannot be distinguished because of the black pigment surrounding the ommatidia.

The techniques used for stimulating and recording are similar to those employed in previous research (Frank and Fuortes, 1956) and will be described in detail elsewhere (Fuortes, 1958 b). It will be sufficient to mention here that a glass micropipette filled with potassium chloride was introduced into cells of the ommatidium and was used both for leading off the potentials generated across the cell's membrane during activity and for passing depolarizing or hyperpolarizing currents through the membrane of the cell. It should be noted that since these currents were passed through a bridge circuit (see Frank and Fuortes, 1956, fig. 1) the changes of membrane potential they evoked were not recorded but could be calculated if the values of current intensity and of membrane resistance were known.

Results

Penetration of ommatidial cells

When a microelectrode is driven into an ommatidium it may suddenly become negative with respect to the outside electrode. Following this, a response to light can usually be recorded although sometime large negative potentials appear but no response to light or to depolarizing currents can be evoked. Only very rarely small responses to light could be recorded in the absence of a negative potential shift (fig. 2-A).

Negativity of the microelectrode accompanied by responses to light is presumed to indicate penetration of an ommatidial cell.

MacNichol (1956) has classified into three types the responses to light recorded with intracellular microelectrodes: (1) small slow potentials and small spikes; (2) large slow potentials without spikes; (3) large spikes with small or no slow potentials.

In the present research very small responses were disregarded and it was found more useful to classify the remaining responses into two groups according to spike size.

Units producing small spikes. In the majority of cases illumination produced large slow potentials and small spikes. The numerous cases in which slow potentials were not accompanied by any detectable spike activity are tentatively included in this group because it was observed that spikes might appear sometime after penetration or following a small movement of the microelectrode. Units belonging to this group are usually penetrated without injury discharge. In some cases the response evoked by a bright light was larger than the potential change recorded upon penetration (fig. 2-B). Spike size ranged in a continuous way between zero and 15 mV.

With good preparations and adequate microelectrodes, penetration of units responding in this manner may occur in over one-half of the explored ommatidia when the electrode is moved down not more than 200 μ from first contact. In some ommatidia more than one of these units was penetrated with a movement of not more than 200 μ.

Units producing large spikes. More rarely, cell penetration (as indicated by a sudden negativity of the microelectrode) was accompanied by high-frequency discharge of large spikes (fig. 2-C). On some occasions this discharge ended with the death of the unit but on other occasions it gradually subsided. Quite frequently the initial high-frequency discharge could be decreased or interrupted by small adjustments of the position of the microelectrode or by hyperpolarizing currents. In a number of instances the conditions of the cell became stable some time after cessation of the initial discharge and

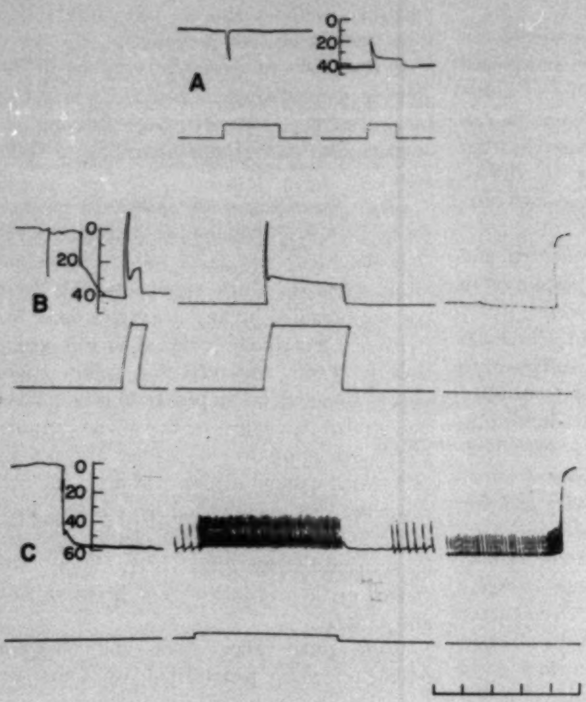

Fig. 2 (Fuortes). Penetration of cells in the ommatidium. (A) Response to illumination recorded in the absence of a membrane potential (left) and after penetration (right). Gain in left-hand record is 3.2 times that of record at right. (B) In the left-hand record the microelectrode is moved down and a cell producing small spikes (not visible in the record) is penetrated. The transient phase of the response to light is larger than the potential shift recorded upon penetration. After a couple of minutes (middle record) membrane potential has increased and the response to light is similar to that recorded just after penetration. In the right-hand record the microelectrode is rapidly withdrawn and membrane potential is lost. (C) A cell producing large spikes is penetrated in the left-hand record. The thickening of the baseline is due to the initial injury discharge. (Note that the spikes appear very small in this record because frequency response of the recording instrument is only 60 c/s.) After 30 minutes (middle record) the injury discharge has subsided and a response to light is recorded. The right-hand record was taken after 30 more minutes, when the cell showed signs of deterioration. Membrane potential is lost upon withdrawal of the microelectrode.

In all records the lower trace is the response of a photoelectric cell.

Time line: one small division equals 1.0 sec.

reproducible responses could be elicited for several hours. Once stable conditions were reached, the size of the spikes obtained from these units was usually over 40 mV. The spikes (fig. 3) present a large hyperpolarizing phase and no rising phase inflection comparable to that recorded from spinal cord motoneurones (Araki and Otani, 1955; Fuortes, Frank, and Becker, 1957; Eccles, 1957). Successful penetration of units producing large spikes occurs only rarely, perhaps once every 50 penetrations of units producing small spikes.

Several features of response were found to be very similar in all cells producing large spikes and a description of some of their properties will be given in this article. Discussion of the responses of cells producing small spikes will be postponed until more experiments are performed and some interpretation of the different findings may be offered.

Responses to light

Generator potential and firing frequency. Typical responses of cells producing large spikes to white light of different intensities are shown in Figure 4. The responses include a transient phase at the onset of stimulation and a steady state thereafter, and consist of (1) a baseline shift indicating sustained depolarization of the cell's membrane and

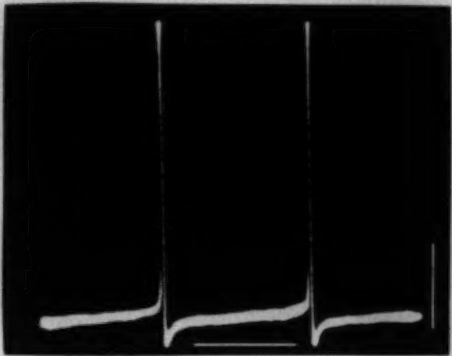

Fig. 3 (Fuortes). Typical impulses recorded from a cell producing large spikes. The hyperpolarizing phase was present in all units belonging to this group. Rising phase inflection was never observed. Calibration: 20 mV. Time line: 100 msec.

(2) repetitive discharge of impulses. Unless the cell fires spontaneously, or is very close to doing so, it can be shown that the depolarization must exceed a certain value before impulses are discharged (fig. 5).

In the presence of firing, accurate measurement of the amplitude of the depolarization evoked by light presents some difficulty but reasonable values can be estimated. In this study measurements were taken only in steady state conditions and amplitude of the slow potential was measured as the distance between baseline and a line traced through the regions where the rate of potential change between successive spikes was slowest. The depolarization evoked by light and measured in this way will be referred to as "generator potential" since this is an accepted term for depolarizing slow potentials of receptor organs.

As shown in Figure 6, both amplitude of the generator potential and frequency of firing are an approximate linear function of the logarithm of light intensity. This observation is in agreement with results obtained by Hartline (1934, 1935, 1940), by recording from optic nerve bundles, and with more recent results obtained by MacNichol (1956), recording with intracellular electrodes from cells producing small slow potentials and small spikes (see his figs. 7 and 9).

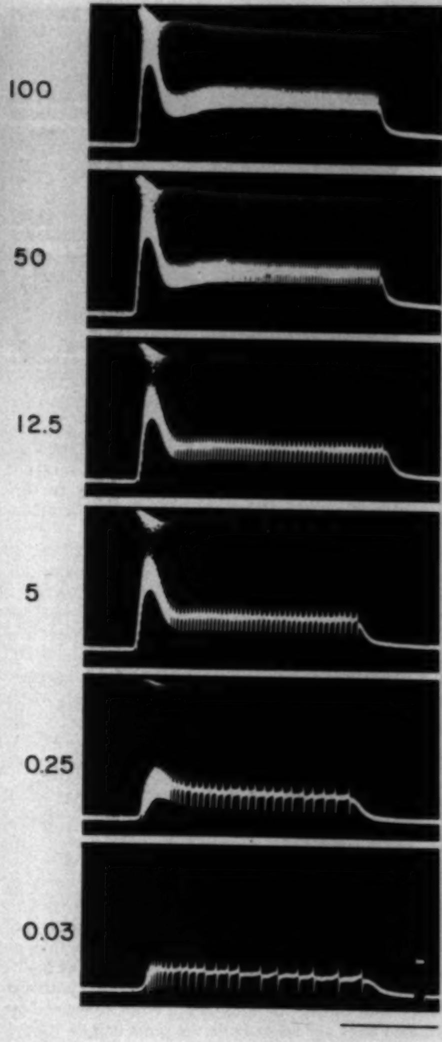

Fig. 4 (Fuortes). Responses to lights of different intensities. The figures at the left give relative intensity of the stimulating light. A small artifact at the beginning of each record shows the time at which the shutter was opened and it can be seen that latency decreases with increasing light intensity. The square wave at the end of bottom record is a 20 mV. calibrating pulse. Horizontal line at bottom indicates 1.0 sec.

Generator potential amplitude and fre-

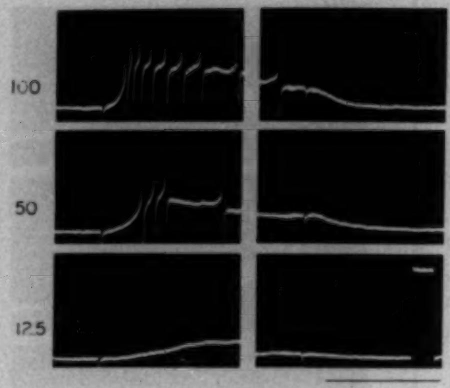

Fig. 5 (Fuortes). Responses to dim lights. Records were taken at high gain so that the major portion of the spikes was off screen. Relative intensity 12.5 was always subliminal for firing but elicited a detectable slow potential. Latency for firing decreases with increasing brightness. Calibration 20 mV. Time line: 1.0 sec.

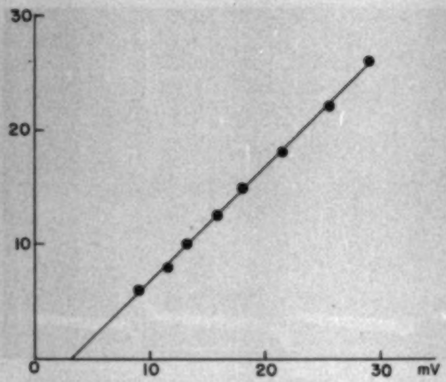

Fig. 7 (Fuortes). Frequency of firing as function of amplitude of "generator potential." Data taken from same unit of Figure 4 but in steady state conditions (15 to 20 sec. after onset of light). Abscissa: generator potential amplitude in mV.; ordinate imp./sec. Frequency and depolarization were changed by stimulation with lights of different intensity. During steady state, frequency is a linear function of depolarization in most units, over a rather extensive range. The slope of this linear function ranged between 0.75 $\frac{\text{imp./sec.}}{\text{mV.}}$ and 4.0 $\frac{\text{imp./sec.}}{\text{mV.}}$ in units producing large spikes.

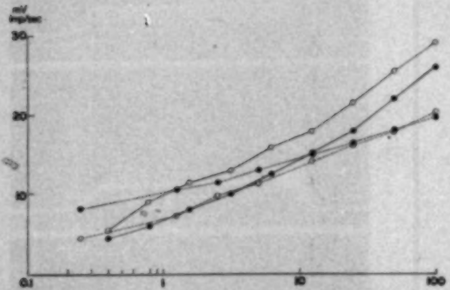

Fig. 6 (Fuortes). Amplitude of "generator potential" and frequency of firing as a function of intensity of stimulating light. Abscissa: light intensity in relative units; ordinate: generator potential amplitude in mV. or imp./sec. Points connected by dashed lines are taken from the same unit of Figure 4. Points connected by solid lines are from another unit. In both units the dots ● are measurements of frequency and the open circles ○ measure the amplitude of the generator potential. Measurements were made between 15 and 20 sec. after onset of light stimulation. Both amplitude of generator potential and frequency are an approximately linear function of logarithm of light intensity for the unit represented by the dashed lines, while for the other unit the relation deviates considerably from linearity. A relation of the form illustrated by the solid lines was found in several other units.

quency of firing were not a strict linear function of the logarithm of light intensity in a number of instances (for example, see solid lines in fig. 5) but they deviated from linearity in the same manner. Consequently, it was observed in most cases that frequency of firing was a close linear function of generator potential amplitude (fig. 7). A similar linear relationship between frequency and depolarization has been found by Katz (1950) in stretch receptors of frogs and by MacNichol (1956) in cells of the Limulus eye producing small slow potentials and small spikes.

Abolition of spikes. A characteristic feature of the responses to illumination obtained in this research is the decrease of spike amplitude which occurs when amplitude of the generator potential and frequency of firing increase (see Fuortes, 1958b). The observation that spike amplitude may drastically decrease in some conditions suggests that, in

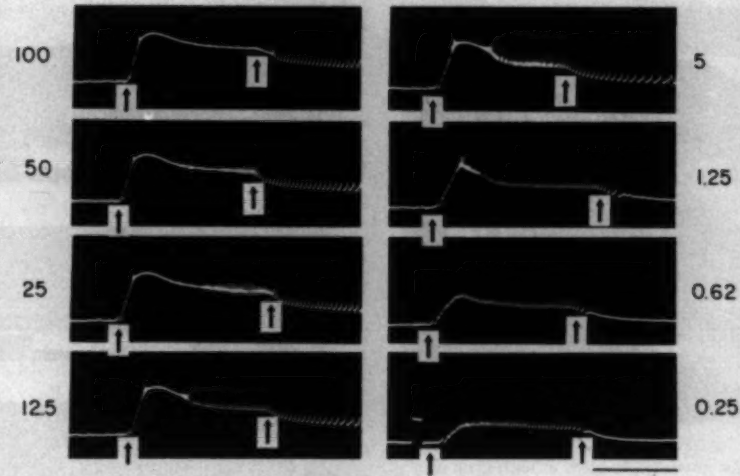

Fig. 8 (Fuortes). Abolition of spikes with high-intensity illumination. Experiment similar to that of Figure 4, in a different unit. The figures near each record give the relative light intensity used for stimulation. The shutter of the light stimulator was closed by hand in this experiment and, therefore, duration of illumination varied in the different records. Arrows indicate the time at which illumination was started and discontinued. Square wave at start of bottom right record is a 20mV. calibration. Horizontal line at bottom indicates one sec. Beam was only moderately intense to avoid overexposure of the baseline. Spikes were therefore too faint for a clear reproduction unless their size was considerably reduced. With intensity 1.25 spikes became very small at peak of transient phase. With higher intensities spikes disappear altogether during increasingly longer fractions of the period of illumination and an "after-discharge" or "off-discharge" is generated. At intensities 25 and 50, an approximately sinusoidal oscillation is superimposed to the "generator potential."

more extreme conditions, spikes may be altogether abolished. This was actually observed in a number of instances and is illustrated in Figure 8. Low intensity of the oscilloscope beam was used for these records, so that the large spike became too faint for reproduction, but a clear picture of the baseline was obtained. It is seen that, following stimulation with bright lights and apparently coinciding with a certain level of depolarization, the spikes are abolished while a roughly sinusoidal oscillation appears. With larger depolarization also the oscillation is abolished. This phenomenon is similar to those previously described by Wiersma, Furshpan, and Florey (1953) and by Eyzaguirre and Kuffler (1955) in invertebrate stretch receptors, by Arvanitaki and Chalazonitis (1955) in aplysia, and by Granit and Phillips (1956) in the cerebellum of cats.

Latency. As previously noted by Hartline (1934, p. 238), the latency of the first impulse decreases with increasing light intensity. In the records obtained in this study (figs. 4, 5, and 9) it is seen that the latency of the first impulse consists of two fractions: (1) time for initiation of the slow potential and (2) time between start of the slow potential and initiation of the first impulse. Although both initiation of the slow potential and transition between slow potential and impulses are gradual processes, it can be seen that both fractions decrease with increasing light intensity.

Therefore, latency for firing is controlled by two different factors and it is not surprising that the relation between latency and light intensity does not have a simple form, as it is seen in the plot of Figure 10.

Responses to depolarizing currents

Current intensity and firing frequency.

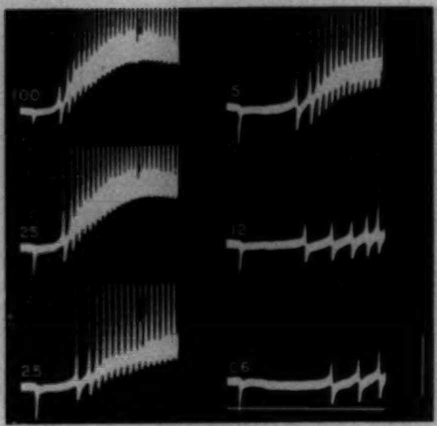

Fig. 9 (Fuortes). Decrease of latency with increasing light intensity. Records at high gain, showing only part of the spikes. The figures at the left of each record give light intensity in relative units. Calibration: 20 mV. Time line: 0.5 sec.

Hartline, Coulter, and Wagner (1952) found that currents flowing in the eye from optic nerve to cornea may evoke impulses in the optic nerve fibers. MacNichol (1956) passed depolarizing currents through the membrane of a cell producing large spikes and found that increase of current intensity by 10^{-9} A increased frequency of firing by about 30 imp./sec.

The results obtained in this study and illustrated in Figure 11 are in general agreement with those quoted above. The records show that electrical stimulation does not evoke a transient initial phase comparable to that evoked by light, but rather it elicits

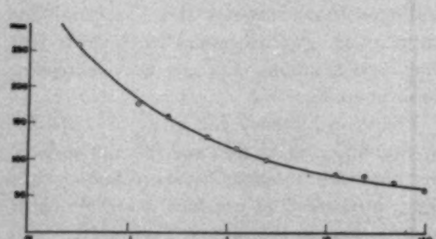

Fig. 10 (Fuortes). Latency of first spike as a function of light intensity. Abscissa: relative light intensity; ordinate: latency in msec.

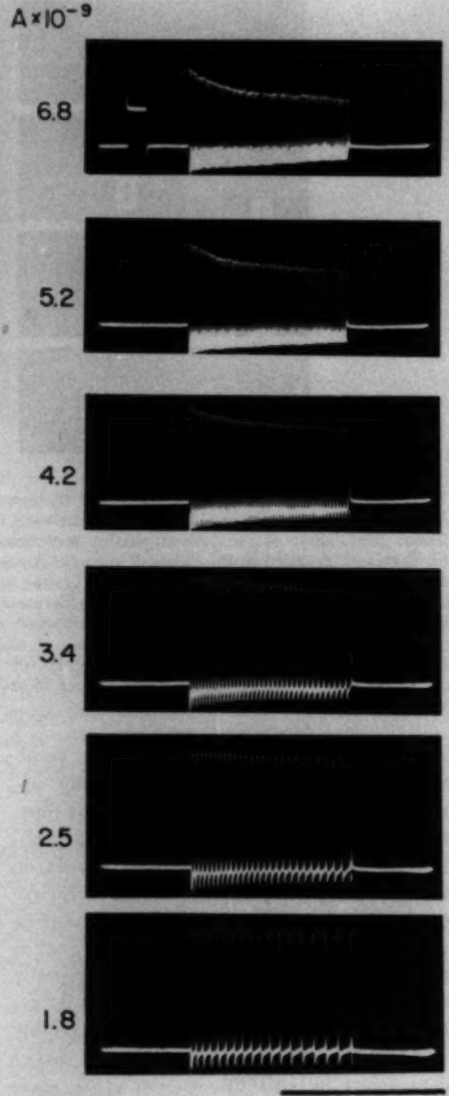

Fig. 11 (Fuortes). Responses to depolarizing currents. Records taken from the unit of Figure 4. Figures at left give intensity of depolarizing current through the microelectrode in A × 10^{-9}. Bridge was slightly unbalanced so that a small downward deflection occurred during the current pulse. Square wave at start of top record is a 20 mV. calibration pulse. Time line at bottom: 1.0 sec.

regular trains of impulses which, during one second, present a slight, progressive decrease of frequency.

According to Hartline, Coulter, and Wagner (1952) frequency of firing increases linearly with the logarithm of current intensity. This logarithmic relation was never observed in the present research and it was found instead (in agreement with MacNichol, 1956) that the relation between frequency at steady state and current intensity is linear over a wide range (fig. 12).

Abolition of spikes. As was the case for firing induced by light, also with depolarizing currents, spike amplitude decreases with increasing frequency of firing and spikes can be altogether abolished following stimulation with high-intensity currents. An example of this phenomenon is shown in Figure 13 where it is seen that, with strong currents, a train of spikes of small amplitude is initiated, but later the spikes disappear. The

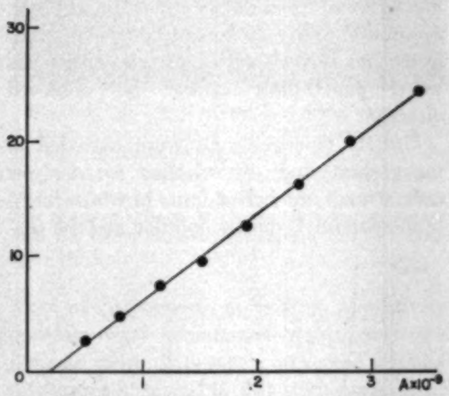

Fig. 12 (Fuortes). Frequency of firing as function of depolarizing current intensity. Data from same unit of Figures 4 and 9, but in steady state conditions. Abscissa: intensity of depolarizing current; ordinate: imp./sec. Frequency was measured between 15 and 20 sec. after onset of constant current. Frequency is a linear function of current intensity in most units, over a wide range. The slope of this linear function ranged between 3.75 $\frac{\text{imp./sec.}}{\text{A} \times 10^{-9}}$ and 25.9 $\frac{\text{imp./sec.}}{\text{A} \times 10^{-9}}$ in the units producing large spikes.

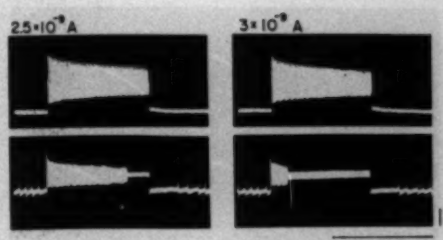

Fig. 13 (Fuortes). Abolition of spikes with high-intensity currents. Top two records show firing induced by depolarizing current pulses of 2.5×10^{-9}A and 3×10^{-9}A respectively. In the two bottom records the same current pulses were applied while the eye was steadily illuminated. In both bottom records spikes are suddenly blocked some time after start of current pulse. Vertical line at bottom right is 20 mV. calibration. Horizontal line at bottom is 1.0 sec.

records are not as clear as those obtained with bright lights (fig. 8) because of the noise generated by strong currents through the microelectrode but the significance of the findings is probably analogous.

Latency. If responses to depolarizing currents are recorded on fast sweeps (fig. 14), the relation between latency of the first impulse and current intensity can be accurately analyzed. As noted by Hartline, Wagner, and MacNichol (1952) latency decreases with increasing current intensity and is always much shorter than it is when a similar discharge is evoked by illumination.

A plot of the relation between latency t and current intensity I (strength-latency curve) is shown in Figure 15, in which the points represent experimental measurements and the solid line is a theoretical curve expressing the relation:

$$I_0/I = 1 - e^{-t/\tau}$$

where I_0 is rheobasic current and τ is the time constant. The good agreement between the experimental points and the theoretical curve (see Fuortes, 1958 a) indicates that the strength-latency relation of cells in the Limulus eye has a form similar to that found in other structures. The time constant τ had values of several (five to 10) msec. in all

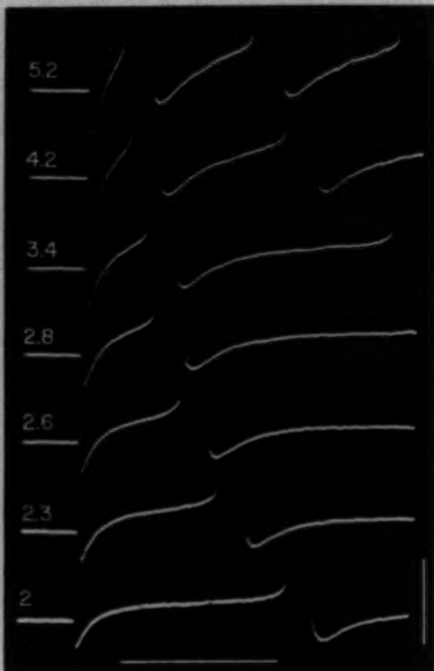

Fig. 14 (Fuortes). Decrease of latency with increasing current intensity. Depolarizing current was applied at the artifact and continued throughout the sweep. Figures at left indicate current intensity in $A \times 10^{-8}$. Calibration: 20 mV. Time line: 20 msec.

cases studied. Therefore, the time constant of the strength-latency curves obtained in this study is considerably longer than that found in other neurone somata (Frank and Fuortes, 1956) with the same technique.

Intervals between successive impulses. Arvanitaki (1938) and Hodgkin (1948), studying the repetitive firing evoked by steps of current in giant nerves of crabs, found that latency of the first impulse has the same order of magnitude as the intervals between successive impulses, up to firing frequencies of about 100 imp./sec. With strong currents, evoking firing at more than 100 imp./sec. latency decreased toward zero but intervals between impulses never became less than 6.5 msec. (Hodgkin, 1948, p. 171).

The properties of cells in the Limulus eye are somewhat different from those of peripheral axons of Carcinus, since in Limulus the intervals between successive impulses are always considerably longer than the initial latency. This is illustrated in Figures 16 and 17 (see for comparison Arvanitaki, 1938, p. 53, fig. 28). The features of firing evoked by current steps in cells of the Limulus eye are instead quite similar to those found in spinal cord motoneurones (Frank and Fuortes, unpublished).

Discussion

Selection of results

Two preliminary difficulties are often encountered when attempts are made to coordinate and interpret results obtained with intracellular electrodes. Since there is ample evidence that impalement of a cell may evoke damage, the first difficulty is to establish criteria for recognizing damage and for selecting the results obtained from the least injured units. Secondly, functional interpretations often require identification of the penetrated unit with known histologic structures and this also may present difficulties when penetration cannot be observed directly.

Grossly damaged cells were excluded in the present study by selecting for analysis only a small number of units in which membrane potential, spike amplitude and the fea-

Fig. 15 (Fuortes). Strength-latency relationship. Data are taken from records such as those of Figure 14. Abscissa: latency for firing of first impulse; ordinate: intensity of depolarizing current. The solid line is a theoretical curve expressing the relation mentioned in text, and has a time constant of 6.7 msec.

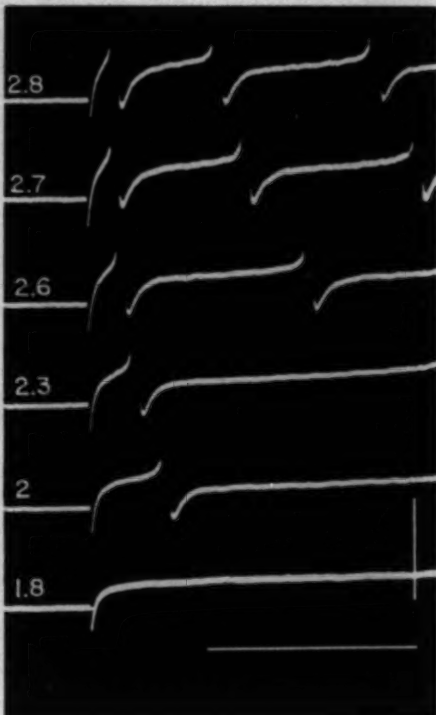

Fig. 16 (Fuortes). Repetitive firing evoked by currents. Depolarizing current was applied at artifact and continued throughout the sweep, as in the experiment of Figure 14. Figures at left indicate current intensity in $A \times 10^{-9}$. The records show that the first interval is always considerably longer than the initial latency. Calibration: 20 mV. Time line: 100 msec.

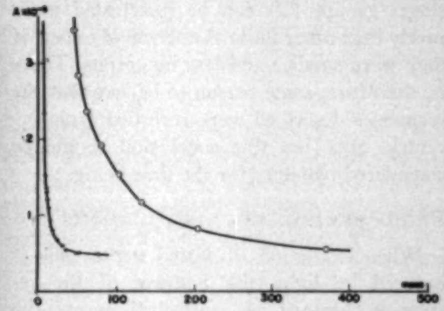

Fig. 17 (Fuortes). Comparison of strength-latency and strength-intervals relations in one unit. Strength-latency (small circles) relation, as in Figure 15. Large open circles show the relation between average duration of intervals at steady state and intensity of direct current used to evoke repetitive firing.

tures of response to light or to stimulating currents were maintained stable over one hour or more. Moreover, since the impulse discharges observed in this study were quite similar to those recorded by Hartline and his co-workers from optic nerve fibers, it is reasonable to think that penetration had not seriously modified the function of the cells selected. It cannot be stated, however, whether some of the finer features of the responses obtained (for example, abolition of spikes with strong depolarization, small amplitude of the recorded spikes, hyperpolarizing phase, and so forth) might be abnormal consequences of the impalement.

Identification of selected units

Concerning histologic nature of the penetrated cells, MacNichol (1956) states that no responses to illumination can be recorded from penetrated retinula cells and suggests that slow potentials without spikes are recorded from the distal process of eccentric cells, large spikes without slow potential from eccentric cell bodies, and small spikes superimposed to a slow potential "from a location in which the electrode intercepted currents from the separate site of origin of slow and spike potentials" (p. 41).

If eccentric cells really were the only elements in the ommatidia producing electric responses to light, then there would be no doubt that the responses described above were recorded from eccentric cells. However, it was observed that penetration of units producing electric responses to light could be easily obtained following penetration of ommatidia in any location. Since the eccentric cell occupies only a small fraction of the volume of the ommatidium, it is probable that light-evoked potential may be recorded from retinula cells, as suggested by Yeandle (1958). In these conditions it can only be said that the units considered in this article (1) present uniform features of response and therefore can be classified in a

single group, (2) can be penetrated more rarely than other units as one would expect if they were smaller and less numerous. There is, therefore, some reason to believe that the responses described were recorded from eccentric cells, but this conclusion should be considered tentative for the time being.

Photochemical and neural events

When excitation of visual nerve cells is induced by light, the features of the responses obtained are controlled in part by the properties of the photochemical processes initiated by light, and in part by processes which subserve generation of impulses in the nerve cell itself. Impulses are generated in the absence of photochemical activity when the impaled cell is excited by electric currents. Thus, comparison of the results obtained with light and with depolarizing current may provide some information on the features of the photochemical activity. However, since the photoreceptors are thought to be external to the eccentric cell (Miller, 1957), and since it is not known whether photoreceptor activity is accompanied by electric changes, records of the electric activity of eccentric cells will give more direct information on the neural events than on the photochemical processes.

Repetitive firing

The repetitive firing evoked by long current steps presents slow adaptation and, in steady state conditions, frequency of firing is a linear function of intensity of the depolarizing current. The method employed did not permit direct measurement of the membrane depolarization evoked by current but it was shown that, when firing is induced by light, frequency of discharge is a linear function of the magnitude of the recorded depolarization. With certain assumptions (to be discussed in a following paper, Fuortes, 1958 b) it can be concluded that membrane resistance remains constant during flow of the applied currents and a measurement of its value may be obtained from these data.

It has been suggested that frequency of firing in response to a constant stimulus is controlled by the time required for recovery from refractoriness (Adrain, 1928 and 1932) or that it is controlled primarily by the time required for building up a fixed amount of depolarization at which firing occurs (Hodgkin, 1948; see also Eccles and Sherrington, 1931); in other words, by the time constant of the system.

If time constant were the only important factor, then the duration of the intervals between impulses should be equal to the latency of the first impulse. This was in fact observed by Arvanitaki (1938, fig. 27) and by Hodgkin (1948) for currents inducing firing of giant axons of crabs at less than 100 imp./sec. In the present study it was found instead that intervals were always longer than latency, as shown in Figures 16 and 17. This observation suggests that refractoriness and accommodation are important in controlling frequency of firing.

If refractoriness were the only factors controlling frequency, then the change of interval duration with changing current should reproduce the course of the recovery cycle. In the only experiments performed to test this point, a spike was elicited by a short shock and recovery of excitability was studied by measuring the strength of a subsequent short shock required to elicit a second spike at various times after the first. Recovery measured in this way was found to be considerably more rapid than the course of the relation between current intensity and interval duration. Qualitatively, these results could have been predicted from the observation that the first interval in a train of impulses elicited by a steady current is shorter than the intervals at steady state. Apparently, the course of refractoriness as conventionally studied does not supply sufficient information for interpreting the relation between stimulus strength and frequency of firing and one should consider the possibility that refractoriness accumulates with increasing number of impulses and that continued

depolarizing currents may have some additional depressant action (accommodation, see Hodgkin, 1951, p. 385). The experiments required to test these possibilities are rather elaborate and have not been performed so far.

Abolition of impulses

Hodgkin (1951, p. 385) and Hodgkin and Huxley (1952) state that the depressant effect of sustained depolarization is due to (1) decreased ability of the membrane to become specifically conductive to sodium ions (sodium "inactivation"), and (2) increased permeability to potassium ions. Several authors (for example, Eyzaguirre and Kuffler, 1955; Granit and Phillips, 1956 a and b) suggested that "inactivation" is responsible for the cessation of firing associated to excessive depolarization of some nerve cells. The firing elicited in visual cells of Limulus by bright lights is often interrupted when the depolarization reaches its maximum peak. The case in which this phenomenon was most clearly seen is illustrated in Figure 7. It is seen in this figure that cessation of the discharge always occurs when the generator potential exceeds a certain amplitude. This observation suggests that excessive depolarization of the impaled cell is responsible for abolition of impulse discharge.

However, Hartline, Wagner, and Ratliff (1936) and Hartline and Ratliff (1957) have shown that excitation of an eccentric cell may inhibit the activity of a neighboring eccentric cell ("lateral inhibition"). Since the bright lights used certainly activated more than one single ommatidium, one should consider the possibility that the transient abolition of impulse discharges described here may be due to "lateral inhibition." There are several reasons for believing that the depolarization of the impaled cell itself is a sufficient condition for abolishing impulse generation. The strongest argument in favor of this view is that spike production can be abolished by depolarizing currents through the impaling microelectrode, since in these conditions no activity is elicited in neighboring ommatidia and "lateral inhibition" cannot occur.

Conclusions

It appears from these results that the photochemical processes initiated by light induce depolarization of the nerve cell membrane (generator potential). Latency, amplitude, and time course (including the transient initial phase) of generator potentials are probably controlled by the properties of these photochemical processes.

The relations between generator potential and impulse discharge are, instead, a consequence of the properties of nerve cells themselves. Frequency of firing is simply related to the amount of depolarization whether this is induced by illumination or by stimulation with electric currents, and impulses may be abolished if depolarization exceeds certain values.

Other properties of the responses of visual cells of Limulus to light and to electric currents will be described in a later article.

Summary

Responses to illumination or to electric currents were recorded from cells of the Limulus eye by means of intracellular micropipettes.

In the majority of cases the response to illumination consisted of a large sustained depolarization with small spikes or no spikes at all. In a smaller number of cases the response consisted of a large sustained depolarization ("generator potential") and large (over 40 mV.) spikes.

Only responses obtained from cells producing spikes of more than 40 mV. were considered in this article. It is presumed that these responses were recorded from eccentric cells.

Amplitude of the generator potential and frequency of firing evoked by illumination are an approximately linear function of the logarithm of light intensity.

Frequency of firing induced by light is di-

rectly proportional to amplitude of the generator potential.

Spike size decreases with increasing frequency of firing and increasing generator potential amplitude. With bright illumination spikes may be altogether abolished whenever generator potential amplitude exceeds a certain value.

Latency of the first impulse decreases with increasing light intensity.

Frequency of firing induced by depolarizing currents is directly proportional to current intensity.

Spike amplitude decreases with increasing frequency of firing and spikes can be abolished following stimulation with currents of high intensity.

Latency decreases with increasing current intensity. The strength-latency curves of different cells had time constants of five to 10 msec.

For long current steps evoking repetitive firing, the intervals between impulses were always considerably longer than the latency for the first impulse.

The results indicate that the photochemical changes initiated by light evoke depolarization of the visual nerve cell and that impulses are generated by these cells as a consequence of the depolarization.

National Institutes of Health (14).

References

Adrian, E. D.: The Basis of Sensation. London, Christophers, 1928.

———: The Mechanism of Nervous Action. Philadelphia, Univ. Pa. Press, 1932.

Araki, T., and Otani, T.: Response of single motoneurons to direct stimulation in toad's spinal cord. J. Neurophysiol., **18**:472-485, 1955.

Arvanitaki, A.: Proprietes rythmiques de la matière vivante. In: Variations graduées de la polarisation et rythmicités. Paris, Herman et Cie, 1938.

Arvanitaki, A., and Chalazonitis, N.: Potentiels d'activite du soma neuronique géant (*Aplysia*). Arch. Sci. physiol., **9**:115-144, 1955.

Eccles, J. C.: The Physiology of Nerve Cells. Baltimore, Johns Hopkins Press, 1957.

Eccles, J. C., and Sherrington, C. S.: Studies on the flexor reflex.—IV. After-discharge. Proc. Roy. Soc. B, **107**:586-596, 1931.

Eyzaguirre, C., and Kuffler, S. W.: Processes of excitation in the dendrites and in the soma of single isolated sensory nerve cells of the lobster and crayfish. J. gen. Physiol., **39**:87-119, 1955.

Frank, K., and Fuortes, M. G. F.: Stimulation of spinal motoneurones with intracellular electrodes. J. Physiol., **134**:451-470, 1956.

Fuortes, M. G. F.: Electrophysiology of the visual system—Discussion. Am. J. Ophth., (in press) 1958 a.

———: Initiation of impulses in visual cells of Limulus. J. Physiol., (in press) 1958 b.

Fuortes, M. G. F., Frank, K., and Becker, M. C.: Steps in the production of motoneuron spikes. J. gen. Physiol., **40**:735-752, 1957.

Granit, R., and Phillips, C. G.: Two types of inhibition of cerebellar Purkinje cells. J. Physiol., **132**: 58-59, 1956 a.

———: Excitatory and inhibitory processes acting upon individual Purkinje cells of the cerebellum in cats. J. Physiol., **133**:520-547, 1956 b.

Hartline, H. K.: Intensity and duration in the excitation of single photoreceptor units. J. cell. & comp. Physiol., **5**:229-247, 1934.

———: The discharge of nerve impulses from the single visual sense cell. Cold Spring Harbor Symp. quant. Biol., **3**:245-250, 1935.

———: The nerve messages in the fibers of the visual pathway. J. optic. Soc. Am., **30**:239-247, 1940.

Hartline, H. K., Coulter, N. A., Jr., and Wagner, H. G.: Effects of electric current on responses of single photoreceptor units in the eye of Limulus. Fed. Proc., **11**:65-66, 1952.

Hartline, H. K., and Graham, C. H.: Nerve impulses from single receptors in the eye. J. cell. comp. Physiol., **1**:277-295, 1932.

Hartline, H. K., and Ratliff, F.: Inhibitory interaction of receptor units in the eye of Limulus. J. gen. Physiol., **40**:357-367, 1957.

Hartline, H. K., Wagner, H. G., and MacNichol, E. F., Jr.: The peripheral origin of nervous activity in the visual system. Cold Spring Harbor Symp. quant. Biol., **17**:125-141, 1952.

Hartline, H. K., Wagner, H. G., and Ratliff, F.: Inhibition in the eye of Limulus. J. gen. Physiol., **39**:651-673, 1956.

Hodgkin, A. L.: The local electric changes associated with repetitive action in a nonmedullated axon. J. Physiol., **107**:165-181, 1948.

———: The ionic basis of electrical activity in nerve and muscle. Biol. Rev., **26**:339-409, 1951.

Hodgkin, A. L., and Huxley, A. F.: The dual effect of membrane potential on sodium conductance in the giant axon of Loligo. J. Physiol., **116**:497-506, 1952.

Katz, B.: Depolarization of sensory terminals and the initiation of impulses in the muscle spindle. J. Physiol., **111**:261-282, 1950.

MacNichol, E. F., Jr.: Visual receptors as biological transducers. In: Molecular structure and functional activity of nerve cells. R. G. Grenell & L. J. Mullins, Editors. Washington: Am. Inst. Biol. Sci., 1956.

Miller, W. H.: Morphology of the ommatidia of the compound eye of Limulus. J. Biophysic. & Biochem. Cytol., **3**:421-428, 1957.

Tomita, T.: The nature of action potentials in the lateral eye of horseshoe crab as revealed by simultaneous intra- and extra-cellular recording. Jap. J. Physiol., **6**:327-340, 1956.

Wiersma, C. A. G., Furshpan, E., and Florey, E.: Physiological and pharmacological observations on muscle receptor organs of the crayfish, Cambarus Clarkii Girard. J. Exper. Biol., **30**:136-150, 1953.

Yeandle, S.: Electrophysiology of the visual system—Discussion. Am. J. Ophth. (in press), 1958.

CONDUCTION VELOCITIES IN RABBIT'S OPTIC NERVE*

AND SOME OBSERVATIONS ON ANTIDROMIC RETINAL SPIKES

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Interest in the problem of conduction velocities in the optic nerve from other than a purely descriptive point of view arose when Chang and Kaada (1950) in the cat found three spike groups of different latencies to which Chang (1952) appointed a role in color reception. He maintained that they conducted specific sensitivities to red, green, and blue in decreasing order of velocity. Later Chang (1956) also found histologically three main groups of fiber diameter with peak sizes of nine to 10, four to five, and one to two μ, respectively. Conduction velocities were given as 70, 30, and 17 m./sec., respectively. P. O. Bishop, Jeremy, and Lance (1953) found two waves only with maximum velocity of 70 and 23 m./sec., respectively.

It is not our intention to pursue this problem and review the subsequent discussion pro and contra the conclusions of Chang. But two recent separate and distinct lines of approach have made us feel that Chang's suggestions should not be discarded too hastily. Lennox (1957) found by microelectrode recording within the optic tract in cats that individual spikes with fast conduction velocities arose from elements with greater sensitivity to red than to other colors. Similarly the spikes that had slow conduction velocity were found to be relatively more sensitive to blue. Recently Ingvar (1956) from a vast amount of data from cerveau isolé cats has demonstrated prominent narrow bands of modulator type—thus restricted to narrow regions of the spectrum—projected onto the cat's visual cortex.

Preliminary to work on the rabbit's visual pathway it was decided to combine some measurements on conduction velocities with microelectrode recordings from the retina of the opened bulb as done in the cat's eye by Granit (1955a, b) and Dodt (1956a). Stimulation in such work is antidromic, to the upper portion of the optic tract, and the discharge is identified as a wave in the optic nerve or as spikes in the retina. Compared with the cat, the rabbit as a preparation has

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the disadvantage that the optic nerve fibers, instead of suddenly losing their myelin sheath at the blindspot, turn nasally and temporally in two thick bundles. Within these the fibers lose their myelin irregularly forming a feather edge terminating in ganglion cells at unpredictable distances from the blindspot. The nervehead lies quite superiorly and is highly excavated. As one proceeds downward with a microelectrode—as we shall show—almost any latent period may be presented at any distance from it. It is impossible to know in casu by what route the antidromic impulse has arrived from the blindspot to the recording point. All the more important it is therefore to possess some measurements of conduction velocities in the optic nerve as a guide to the retinal findings. The two types of data supplement each other.

G. H. Bishop (1933) measured conduction velocities in the rabbit's optic nerve having made very extensive operations to lay it bare. He found two waves and the first one varied in conduction velocity as much as from 50 to 20 m./sec. There was no definite slow wave comparable to the one he had seen in the bullfrog. Bishop himself did not seem satisfied with this result. Theoretically his technique of setting up the optic nerve as a peripheral nerve should be set up for measurements of conduction velocities seems correct and ideal, but for biologic reasons we have preferred to use the stereotaxic Horsley-Clarke method of stimulation.

Procedure

Rabbits were given urethane or prepared by the encéphale isolé method of Bremer (1936). The head was placed in the Horsley-Clarke stereotaxic apparatus and the roof of the skull opened. A drop of tetracaine solution was instilled into the right eye. A cannula was inserted into the femoral vein for later injections of the curarizing substance Flaxedil to prevent eye movements.

Stimulating electrodes were concentric needles inserted into the lateral geniculate body or upper portion of the optic tract. For measurements of conduction velocities, one silver pin was stuck into the optic nerve through a retrobulbar cut, another inserted in the soft tissue covering the bulbar cavity. On the bulbar side the nerve was crushed. Both electrodes were conducting only at the tips. Retinal spikes were recorded by micropipettes filled with three molar sodium chloride solution and kept at resistances around a few megohms (Fatt, 1957). A circuit for checking electrode resistance from time to time was introduced. Cathode follower, amplifiers, and oscillograph were employed in the customary way.

Results

1. Optic nerve and tract

In Figure 1 record A1 is exceptional in that in this case four entomologic pins, insulated except at the tip, were thrust into the optic nerve at interelectrode distances of 1.5 mm. Stimulation took place between pins 1 and 2, recording between pins 3 and 4. The picture is superimposed DC records at high sweep speed. The shock artefact is seen to end on the rising phase of the response.

Records A2 and 3, at different sweep speeds, show the characteristic shape of the wavelets obtained for full conduction distance (table 1). Nearly always the first big wave is split up into three humps. If these are not seen from the beginning slight adjustment of the stimulating electrodes generally suffices to make them visible. With stronger stimuli, such as used in the instances illustrated, a delayed, flattened wave follows the early better synchronized responses. At the slower sweep speed in A3 the delayed wave is better set off against the baseline. This record was taken at high sensitivity of the amplifier and was one of the many fruitless attempts to find later waves than the ones seen. When such later waves occasionally were noticed they did not appear in superimposed records and hence were due to casual variations. All records A are taken with DC amplification.

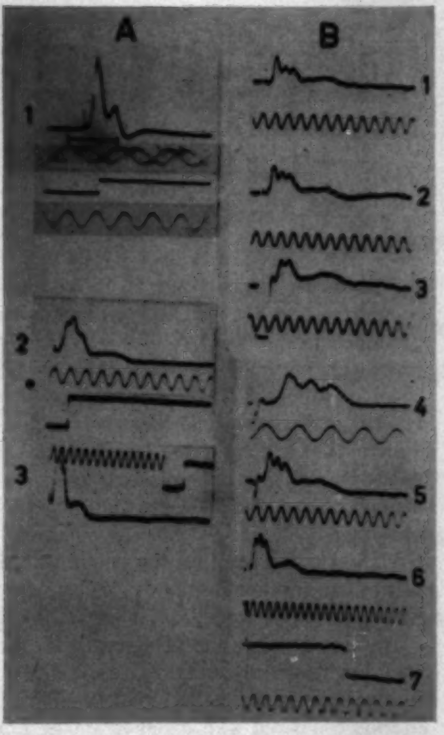

Fig. 1 (Granit and Marg). (A) DC records. (1) Four insulated steel pins with free tips in a row, 1.5 mm. interelectrode distance, stuck into optic nerve between chiasma and foramen opticum. Stimulus between 1 and 2, recording between 3 and 4. (2) Standard leads from retrobulbar end of optic nerve, concentric needle electrode placed by Horsley-Clarke instrument in lateral geniculate body or upper end of optic tract for delivery of single shock initiating sweep. DC calibration to 300 μV. (3) Same but sweep speed reduced and amplification increased (calibration to 100 μV in inset) in order to look for later waves. None visible. (B) AC records with standard leads and slow condensers, as shown by calibration to 100 V in 7. Shock shifted inward on sweep. (1) Brief shock. (2 and 3) Stimulating electrode withdrawn 0.5 mm. Longer shock duration in 3 emphasizes late wave. (4 to 6) Another experiment shown on three sweep speeds. This experiment ended with section of optic nerve by a thin scalpel cutting through chiasma. The wavelets disappeared. All times in msec.

The records B were obtained with large condensers, as shown by calibration in B7. B1 differs from B2 and 3 merely by a slight vertical shift of the stimulus electrode. B2 and 3 compare a short with a long stimulus duration. The latter emphasizes the delayed wave. The first wavelet is distorted by the stimulus artefact. Records B4 to 6, from another experiment, show the same response at three different sweep speeds. The fast wavelets have also been seen by Noell (1953).

The delayed wave, as stated, required stronger shocks than the early, well synchronized portion of the response and experiments with double shocks showed it to have a longer refractory period than the others. In one experiment we successfully injected a drop of two-percent procaine solution into the chiasma and found the slow wave to disappear before the other ones. In another experiment a thin scalpel was used to cut the chiasm from above. All waves then disappeared instantaneously. Results of this type have been grouped together in Table 1 for which the conduction distances were measured immediately after the experiment. For this purpose the optic foramen was opened and the brain tissue cautiously aspirated without moving the electrodes so that the optic tract and nerve lay bare for inspection and measurement. Latency was measured to the beginning of each early wavelet and the delayed wave.

Conduction velocity of the fastest spike varied between 49 and 65 m./sec. with an average value of 56 m./sec. The fastest wave is thus slower than in the cat (see above; 70 m./sec.). Very regular was also a wavelet with conduction velocities varying between 21 and 24 m./sec., average 23 m./sec. Equally regular was the delayed wave with a conduction velocity from 9.4 to 11 m./sec., average 10 m./sec. The third fast wavelet mostly appeared at 16 m./sec., but in two cases, when this was absent, the third fast wavelet occurred at respectively 32 and 38 m./sec. All these are maximum values.

The stimulating needle will, of course, sample bundles selectively (Noell, 1953; Chang, 1956) but there can be no doubt about the existence of groups of different conduction velocities in the fast range nor is

TABLE 1

Conduction Distance (mm.)	Velocity (m./sec.)	Velocity (m./sec.)	Velocity (m./sec.)	Velocity (m./sec.)	Velocity (m./sec.)
20	57		23	16	11
31	52	38	23		9.9
32	49		22	15	9.4
32	56	32	21		10
26	65		24	16	10
AVERAGE	56	32–38	23	16	10

the existence of the delayed wave in the least doubtful. It was seen in every case and the variation in conduction velocity was small. Once, a still later wave conducted at 7.6 m./sec. was seen to rise on top of the slow wave. The most regular faster wavelets are apparently represented by bundles of considerable size containing many fibers of approximately the same diameter.

2. RETINAL RECORDS

In spite of the difficulties of determining intraretinal conduction distance it was deemed necessary to make an attempt at verifying the existence of spike groups of different conduction velocities also by recording with microelectrodes from retinal ganglions. While in the cat a very precise value can be obtained at the blind spot (Granit, 1955a) and the spikes immediately slow down outside it owing to demyelination (see the later measurements of intraretinal conduction velocities by Dodt, 1956b), it is not feasible in the rabbit to make similar measurements. Firstly, the blind spot does not seem to respond. Secondly, demyelination is irregular.

In some measurements we found that the conduction distance from the retrobulbar tip to the blind spot was about 6.0 mm. (the nerve runs for a brief distance alongside the bulb and the tip of the retrobulbar electrode cannot be placed immediately opposite the blindspot). At rate 56 m./sec. and 6.0 mm. between the blindspot and the retrobulbar electrode the earliest spikes should be recordable at the blindspot at a latency of about one-half msec. (table 1). Actually, however, our shortest values have been 1.7 msec. and the commonest ones around 2.0 msec. Thus the micropipette has not recorded any spike from the blindspot itself.

Samples of microelectrode records are shown in Figure 2. Only records from the two ends, superior or near blindspot, and inferior or as far away from it as practicable (about 8.0 mm.), have been selected for publication because these best illustrate the salient point, namely that distance from the blindspot is less significant than the joint effect of unknown demyelination and similarly unknown circuitous route of individual fibers as they emerge from the feather edge of the nasal and temporal bands of massed fibers. However, nerve spikes, which are of brief duration compared with ganglion spikes, are oftener seen near the blind spot than elsewhere. Samples are shown in A1 and E1. Because of the large gaps between the ganglion cells the thin microcapillaries sometimes pass through the ganglionic layer without recording spikes. If advanced too far, the electrode may break its tip against the sclera. It was therefore found advantageous to keep a continuous record of the DC level as well as of the response caused by a flickering light stimulus. The spikes, elicited by this light, signal the ganglionic layer by on/off-discharges heard in the loudspeaker; the DC control gives a big shift of potential somewhere inside the retina, possibly Brindley's (1956) so-called R-membrane. When this happens, electrode has been advanced too far.

The characteristic picture obtained when no attempt is made to localize individual ganglion cells is shown in A2 and F3. There is an early grouped response, a silent period

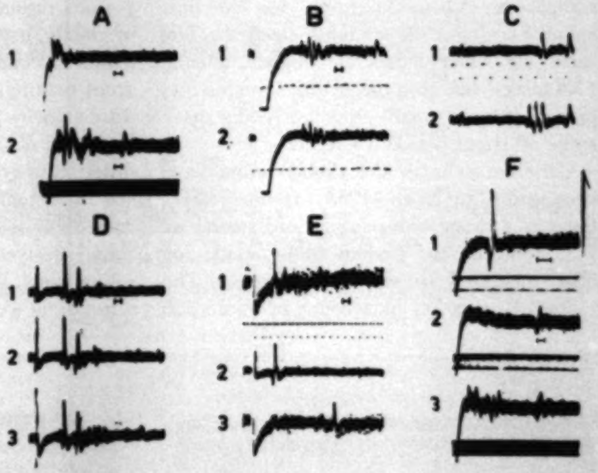

Fig. 2 (Granit and Marg). Six experiments—A-F. Microelectrode records from rabbit's retina responding to antidromic stimulation of optic tract. Superimposed sweeps. Time in msec., marked by vertical lines, except in C where time marker shows up despite superposition. (A) (1) Superior, briefest latency 1.7 msec. Strength 6.4 V; (2) inferior, early spike at 2.4, late at 10.6 msec. Strength 20 V. (B) Inferior, strength 6.4 V; (1) early spikes at 2.5, late spike at 16 msec.; (2) same after withdrawing electrode 30 µ upward. (C) Superior (1) latency 5.5 msec., strength 1.6 V; (2) latency 4.6 msec., strength 2 V. (D) Inferior (1) latency 3.6 msec., strength 0.26 V; (2) same, 0.4 V; (3) same at 1.2 V, latency of brief, earliest deflexion 3.1 msec. (E) Superior (1) at threshold 0.4 V (note fast nerve spike), latency 2.8 msec., high gain; (2) slight movement to isolate cell response, decrease of gain and increase of strength to 2 V; (3) adjacent place, strength 2 V, late spike at 11.0 msec. latency. (F) Inferior (1) latency 2.7 msec., strength 3.2 V; (2) slight lateral shift of microelectrode, latency 11.0 msec., strength 16 V; (3) increased gain, strength 5 V. Big spike in F1 is 380 µV, small spike in F2 140 µV. B, C and D are *encéphale isolé* preparations.

and a delayed spike which always is small. Series D is taken at increasing stimulus strength to show that the delayed small spike as a rule requires stronger shocks. The microelectrode is located between two fairly well isolated cells. With stronger stimuli more distant cells give responses filling out the gaps between the better isolated ones. The latency of the delayed wave is 13.6 msec.

Such findings raise the question of whether or not the delayed response is due to repetitive firing. Two lines of evidence show that it really is an individual spike responding once. In B1 it is present, in B2 absent. These records merely differ by a shift upward of 30 µ on the vertical scale of the micromanipulator. The small delayed spike was seen at the greater depth. In the records F a large early spike was first isolated. Then the delayed spike was found by a small lateral shift of the microelectrode. (Note change of time base.) By moving the stimulating electrode and adjusting strength of the shock it proved possible to obtain the delayed wave in perfect isolation (F2). Another similar experiment is illustrated in series E. In this case, exceptionally, the small delayed spike was found in the superior portion of the retina (E3). More often the small late spikes are located to the middle or inferior part of the eye. Thus the small delayed spike is an ~~individual~~ late response from a small cell activated by high threshold fibers and not a repetitive dis-

charge of cells at some distance from the electrode.

Finally records C show with large spikes that near the blind spot latencies as long as 4.6 and 5.5 msec. can be obtained. The latencies in the records D (inferior) were by comparison 3.1 to 3.6 msec. for the first spike, varying with stimulus strength. The early big spike occurs at constant latency (3.6 msec.) but with the strong stimulus it is preceded by a small—apparently distant—spike of latent period 3.1 msec.

Ganglion cells in the rabbit retina have been studied by Noell (1953). In his pictures there is a range of variation in diameter of 1:3. Some of our Bodian stains, made for other purposes, are shown in Figure 3. The range of variation in a count of some 350 cells was from 4.0 to 12 μ with a broad peak around 7.0 to 8.0 μ. Great accuracy cannot be claimed for these figures as the nuclei could not been seen in the thick Bodian sections and the amount of shrinkage is unknown. It was also noted that ganglion cells occurred at various retinal depths (Polyak, 1941). It is, of course, not possible in physiologic experiments to deduce the actual depth of the cell from readings on the micromanipulator, the distortion by pressure being unknown. The histologic observations were made merely in order to ascertain that large variations in the size of the ganglion cells actually do occur.

In view of the fact that Dodt (1956a) has found delayed small spikes in the rabbit's retina which he by various criteria held to be centrifugal we occasionally tried two of those

Fig. 3 (Grant and Marg). Four microphotographs from different parts of rabbit's retina. Bodian stains 15 μ thick. Layer of ganglion cells downward in all pictures. (×500.)

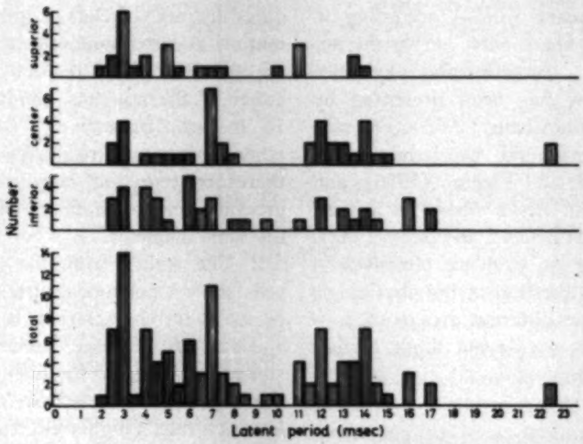

Fig. 4 (Granit and Marg). Histogram of the latency distribution of antidromic retinal spike groups from stimulation of the lateral geniculate body in the rabbit. Measurement was made to the beginning of the initial negative wave in each group of retinal spikes. Single repeatable spikes were measured when they were separate and distinct even though they were not part of a group. Hence this histogram tends to minimize the peaks of the distribution, yet it clearly shows a polymodal character. Latencies measured from the superior, central, and inferior retina indicate an increasing latency which is probably because of the longer, slow neural path as one goes down the retina from the optic nervehead. However the effect is not clear cut in the rabbit because the nerves are irregularly medullated for some millimeters across the fundus.

criteria, light adaptation and stimulation at faster frequencies, to find out if the delayed spikes then disappeared as did Dodt's centrifugal spike. Every small and late spike was not studied in this manner but we proved to our satisfaction that some, at least, neither were influenced by light adaptation nor by increasing rate of stimulus repetition up to frequencies considerably beyond the values found by Dodt to block his centrifugal spikes. As a whole, then, our evidence goes to show that most of the delayed small spikes are due to ordinary centripetal fibers of high threshold being stimulated antidromically. These fibers are therefore likely to take their origin in the small ganglion cells of the retina.

Our results are displayed in the histogram of Figure 4, fully explained in its legend. The distribution is clearly polymodal and the early spikes, too, show preferential distributions. In the group of delayed spikes there may well be some belonging to Dodt's category.

Comment

Our results clearly show that impulses in the rabbit's optic tract and nerve travel in fibers of characteristically grouped conduction velocities, thus introducing a time factor in the excitation of higher stations. Such findings necessarily raise questions of functional differentiation, both with regard to entrance and end stations. We have already mentioned as one possibility the recent work on color discrimination in terms of conduction velocities. Functional differentiation may, however, concern any other aspect of the complex visual message. Particularly interesting is the slowly conducting system from small ganglion cells, likely to possess narrow dendritic fields. These are likely to belong to cones (Ramon y Cajal, 1894; Polyak, 1941). Noell (1953) also points out that the predominance of small ganglion cells in the central areas of most species in combination with the fact that most optic nerve fibers are small is contradictory to a generalization of

Bishop and O'Leary (1938) according to which the small fibers only run to the superior colliculi. Experimental evidence against this view has been presented by Granit (1955b) and Chang (1956). The rabbit has some peripheral discrimination of wavelength; Dodt and Elenius (1956), and has 265,000 optic nerve fibers as against 119,000 in the cat (Bruesch and Arey, 1942). However, having no evidence ourselves to contribute to the question of the physiologic significance of the different groups of conduction velocities, we do not think further speculation justified.

Summary

By the Horsley-Clarke stereotaxic technique stimuli have been applied to the rabbit's optic tract and the response to each shock recorded (1) at the crushed retrobulbar region of the optic nerve and (2) from retinal ganglion cells by NaCl-filled capillaries with tips of the order of 2.0 to 5.0 μ.

1. The optic path (tract and nerve) conducts distinct wavelets of grouped action potentials at maximum velocity of 56, 35, 23, 16, and 10 m./sec., respectively. Sometimes either of the wavelets, conducted at 35 and 16 m./sec., respectively, are absent. The other components are always found and must therefore represent large relatively homogeneous bundles of fibers of approximately the same diameter.

2. The spikes from the retinal ganglion cells show a polymodal distribution of latent period. Very characteristic is a delayed, small spike (11 to 15 msec. latency) which is assumed to belong to the fiber group conducting with maximum velocity of 10 m./sec. It has, as a rule, a higher electric threshold than more rapidly conducted spikes. Small retinal ganglion cells have been found from which thin, slowly conducting fibers would be likely to arise.

Acknowledgment

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References

Bishop, G. H.: Fiber groups in the optic nerve. Am. J. Physiol., **106**:460-474, 1933.

Bishop, G. H., and O'Leary, J.: Potential records from the optic cortex of the cat. J. Neurophysiol., **1**:391-404, 1938.

Bishop, P. O., Jeremy, D., and Lance, J. W.: The optic nerve. Properties of a central tract. J. Physiol., **121**:415-432, 1953.

Bremer, F.: Nouvelles recherches sur le mécanisme du sommeil. Compt. Rend. Soc. Biol., **122**:460-463, 1936.

Brindley, G. S.: The passive electrical properties of the frog's retina, choroid, and sclera for radial fields and currents. J. Physiol., **134**:339-352, 1956.

Bruesch, S. R., and Arey, L. B.: The number of myelinated and unmyelinated fibers in the optic nerve of vertebrates. J. comp. neurol., **77**:631-665, 1942.

Chang, H. T.: Functional organization of central visual pathways. Proc. A. Research Nerv. Ment. Dis., **30**:430-453, 1952.

———: Fiber groups in primary optic pathways of cat. J. Neurophysiol., **19**:224-231, 1956.

Chang, H. T., and Kaada, B.: An analysis of primary response of visual cortex to optic nerve stimulation in cats. J. Neurophysiol., **13**:305-318, 1950.

Dodt, E.: Centrifugal impulses in rabbit's retina. J. Neurophysiol., **19**:301-307, 1956a.

———: Geschwindigkeit der Nervenleitung innerhalb der Netzhaut. Experientia, **12**:34, 1956b.

Dodt, E., and Elenius, V.: Spektrale Sensitivität einzelner Elemente der Kaninchennetzhaut. Pflüg. Arch. ges. Physiol., **262**:301-306, 1956.

Fatt, P.: Electro potentials occurring around a neurone during its antidromic activation. *J. Neurophysiol.*, **20**:27-60, 1957.

Granit, R.: Receptors and Sensory Perception: A discussion of aims, means, and results of electrophysiological research into the process of reception. New Haven, Yale Univ. Press, 1955a, pp. vii + 369.

———: Centrifugal and antidromic effects on ganglion cells of retina. J. Neurophysiol., **18**:388-411, 1955b.

Ingvar, D. H.: Comparison of spectral sensitivity curves from the retina and central visual pathways in the cat. XX Internat. Physiol. Congr., Brussels, 1956, pp. 459-460.

Lennox, M. A.: Responses in single optic tract fibers of cat to monochromatic light: Correlation with conduction velocity. Acta physiol. scand., **42** (suppl. 145):94-95, 1957.

Noell, W. K.: Studies on the electrophysiology and the metabolism of the retina. USAF School of Aviation Medicine, Project No. 21-1201-0004, report no. 1, 1953, p. xi + 122.
Polyak, S.: The Retina. Chicago, Univ. Chicago Press, 1941, p. 607.
Ramon y Cajal, S.: Die Retina der Wirbeltiere. Wiesbaden, Bergmann, 1894, p. 168.

THE ELECTRIC ACTIVITY OF THE EYE DURING ACCOMMODATION

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INTRODUCTION

The physiology of the accommodative process has been studied by a variety of techniques. The most widely held theory of the mechanism of accommodation is that of Helmholtz, as modified by Gullstrand and, most recently, Fincham.[1] This concept states that the activity of the ciliary muscle in the accommodative process allows the deformation of the soft lens substance by the lens capsule in such a manner as to compress it at the equator, where the capsule is thickest, and allow it to become thicker axially, where the capsule is thinnest.

The evidence upon which this theory is based is primarily that of the change in shape of the lens, as measured by Purkinje images, by the variation in form of the capsule of a shrunken lens, and the distance of the anterior surface of the lens from the cornea at various stages of accommodation. Recently, Glezer and Zagorulko,[2] in Russia, have used a photomultiplier circuit to obtain an objective record of the variation in curvature of the anterior lens surface. The recordings obtained reveal a slow rate of change, leading to a plateau of a certain level, with some wavelike variations along the course of the curve. Approaches to the study of the physiology of the ciliary muscle itself have been less numerous. One of these, the work of Melton, Purnell, and Brecher,[3] dealt with the mechanical changes in the muscle as produced by stimulation of the sympathetic and parasympathetic nerves. A mechanoelectric transducer was connected to the ciliary body and accurate record of the contraction of the muscle in the several planes was obtained. These results also were a slow type of change, with a plateau after a period of time.

It has been our intention to apply the techniques of electrophysiology to the ciliary muscle and its function, in an attempt to determine its characteristics. Our study has proceeded along three main channels.

1. Microelectrode study of excised animal ciliary muscle.
2. Macroelectrode study of excised and in vivo animal muscle.
3. In vivo study of human eyes in the course of accommodation.

1. MICROELECTRODE STUDIES OF EXCISED ANIMAL CILIARY MUSCLE

Buhlbring[4] has shown, using intestinal smooth muscle of animals, that spike potentials are generated in smooth muscle cells in response to the stimulation by mechanical stretching of a strip of tissue. Brune and Kotowsky[5] have found similar changes.

In our study a strip of ciliary body was excised from the eye of a cat or monkey under pentobarbital anesthesia. The ciliary body was dissected free of the underlying sclera while immersed in a saline bath, and suspended between a clamp and a transducer which recorded the amount of tension on the muscle strip.*

The apparatus was so arranged that the degree of tension could be varied by motion of a lever. A micropipette electrode, about one micron in diameter, was inserted into the center of the strip of muscle so suspended.

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* Grafax Instrument Co., New York.

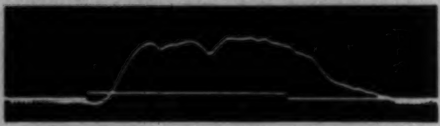

Fig. 1 (Jacobson, et al.). Curve of change in light reflex from human lens in accommodation. (From Glezer and Zagorulko.[3])

The electrode was connected to the input of a transistorized DC differential high impedence input amplifier. A dissecting microscope was used for positioning the electrode.

It was found that in a muscle suspended under little or no tension, an occasional spike potential was generated. Upon stretching the muscle strip, a series of spikes was produced, following which the tissue regained quietude and, upon release of the tension, a new set of spikes was recorded.

The rate of the spike discharges varied from one preparation to the other, but were generally of the order of two or three per second. They varied from 50 to 80 millivolts in amplitude. Four percent procaine solution applied to the muscle immediately stopped all spikes. Histologic verification of the area under study was performed, to insure accuracy of dissection.

2. Macroelectrode studies of excised and in vivo animal muscle

Following ether anesthesia, the ciliary ganglion of cats was exposed by removal of part of the lateral wall of the orbit and dissecting along the course of the nerve to the inferior oblique, toward the apex of the orbit. The ganglion, thus exposed, was suspended upon two stimulating electrodes. A needle electrode was inserted into the ciliary muscle through the sclera. In some instances a strip of sclera was excised over the ciliary area and a 1.0 mm. disc electrode was sewn in place.

The eye was so fixed that no motion of the globe could occur. In most instances all extraocular muscle insertions were severed. Stimulation of the ciliary ganglion with 200 pulses per second from a square wave generator, for periods of three to 10 seconds, was performed.

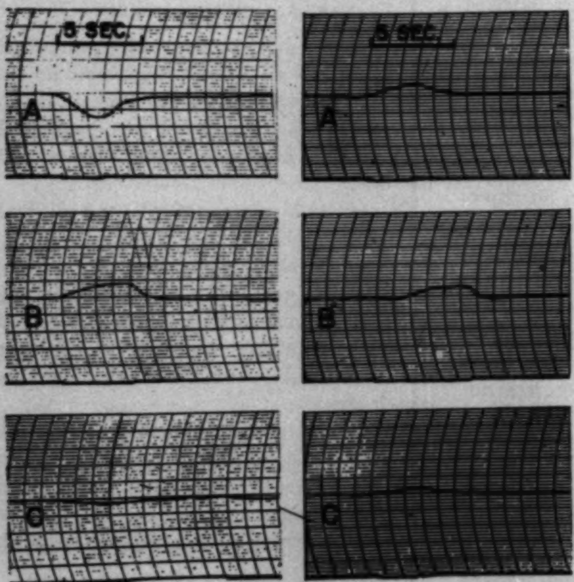

Fig. 2 (Jacobson, et al.). Curves at mechanical changes in ciliary body upon sympathetic stimulation. (From Melton, Purnell, and Brecher.[8])

Fig. 3 (Jacobson, et al.). Experimental setup for micropipette recording of intracellular potentials from ciliary muscle, showing dissecting microscope, electrode in holder, and clamps used to alter tension upon muscle strip.

A slow potential change was recorded from the ciliary body area following stimulation. This potential was present after excision of the iris. The difficulty of the dissection of the ciliary ganglion and the severe trauma to the area which the dissection usually produced, as well as the artifacts in the recordings from the ciliary body area due to the proximity at the stimulation electrodes, led us next to attempt stimulation of the third nerve nucleus area of the brain as a means of inducing accommodative effect in cats.

The cats were immobilized under ether anesthesia and the skull opened with a drill. A stimulating electrode was placed under stereotactic control in the area of the third nerve nuclei. Stimulation with one second bursts of square waves of 50 to 200 cycles per second, of 0.6 volts, produced changes in the retinoscopy images of three or four diopters. Direct observation of the lens through a slitlamp, and photography at the image of the lens, was performed and a significant thickening of the lens was observed.

A slow potential of the type previously discussed was recorded from the area of the ciliary body during stimulation.

In another group of experiments a strip of cat ciliary muscle was suspended between a rigid support and a bridge type transducer. The output of the transducer was recorded in the course of applying electric stimulation in the muscle. A slow contraction of the muscle, comparable to the duration of the slow electric discharge, was recorded.

In another series of experiments the extraocular muscles of cats were severed, and the cats immobilized with succinyl choline. Objects, varying from lights to hands to bits of

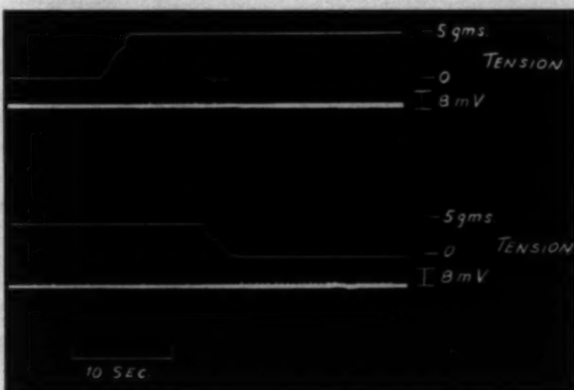

Fig. 4 (Jacobson, et al.). Spike potentials recorded from ciliary muscle fibers. Upper tracing in each record measures tension upon strip as recorded by transducer; lower record, electric changes in muscles. Bursts of activity produced by both increasing and diminishing tension.

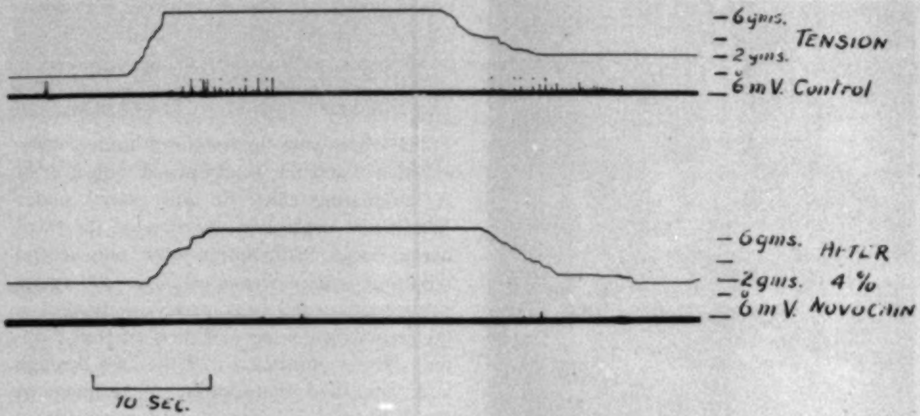

Fig. 5 (Jacobson, et al.). Abolition of spikes by Procaine.

food, were brought into the view of the animal at close range. Potential changes of a slow, periodic character were frequently elicited.

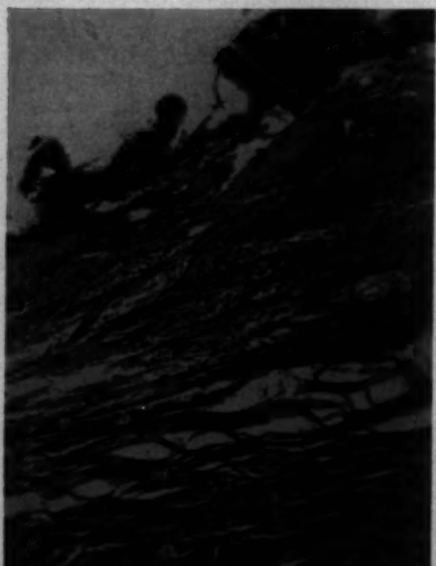

Fig. 6 (Jacobson, et al.). Histologic preparation of area used for microelectrode studies.

3. Human experiments

A. Prior work

The first paper on the subject of electric potential changes in the human eye during accommodation appeared in 1955, written by Schubert.[6] His findings disclosed that in humans, during accommodation upon a near target, a slow potential could be recorded. He used a contact lens electrode, from which the corneal portion had been removed. Pupillary contraction induced by consensual light stimulation did not produce changes of the type recorded as a result of accommodation.

B. Studies

Method 1

Our experiments consisted of recording from the eyes of human volunteers during accommodation, using several different techniques. All recordings were performed inside a shielded, grounded chamber. The subject was seated comfortably at an optical instrument table with head and chin rest. At 25 cm. from his eye a large one way mirror was positioned at a 45-degree angle to the frontal plane of the subject. A target, consisting of a self-illuminated 20/70 size Landoldt ring, was located four meters from the eye. Immediately behind the mirror a self-illumi-

Fig. 7 (Jacobson, et al.). The effect of high frequency stimulation of the ciliary ganglion on the DC potential of the unanesthetized, succinylcholine paralyzed cat eyeball.

nated target, usually a maltese cross, was so situated that it was about 28 cm. from the eye. Prior to recording, both targets were illuminated and their positions so arranged that the cross was superimposed upon the ring, in order to eliminate, as much as possible, motion of the globe upon shift of fixation from one to the other.

The test area was darkened so that neither target could be seen if it were not illuminated separately. The illumination of the targets were controlled by remote switches at the recording instruments, and could be varied in intensity and duration. Either or both targets could be illuminated at will. A battery operated light source was used to eliminate artifacts due to lighting current.

Several types of electrodes were used, including an electroretinographic contact lens, a fenestrated lens as suggested by Schubert, cotton balls soaked in saline and held at the sclera by a spring, and electrodes on the skin at the rim of the orbit.

The potentials were fed into the input of direct coupled amplifiers (Grass P-5 and American Electronic Laboratories No. 131) and the condenser coupled amplifiers of a Grass Model 3 electroencephalograph machine. Recordings were primarily done with the ink writer, although occasional tracings were recorded from the cathode ray oscillo-

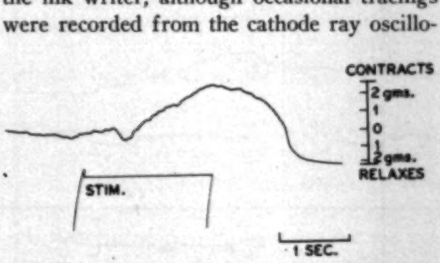

Fig. 8 (Jacobson, et al.). Mechanical changes in strip of ciliary muscle upon electric stimulation of the muscle.

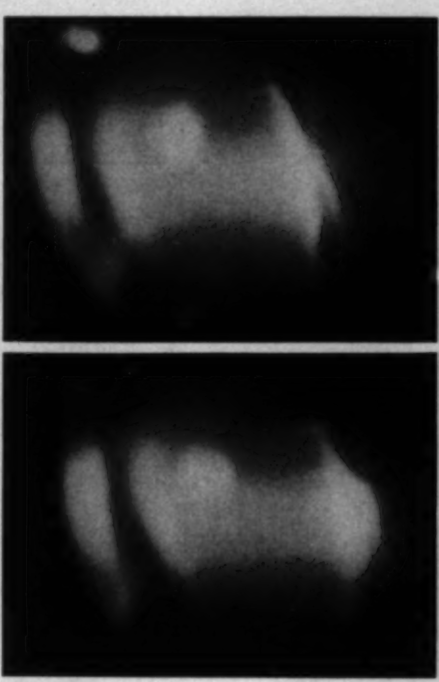

Fig. 9 (Jacobson, et al.). Slitlamp section of cat lens. (Above) Before electric stimulation. (Below) After electric stimulation of third nerve nucleus. Anterior, left.

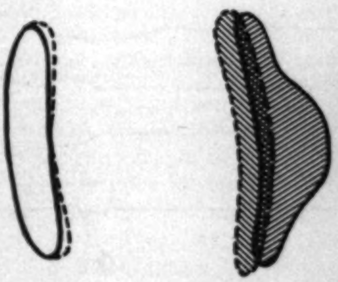

Fig. 10 (Jacobson, et al.). Artist's reconstruction of corneal and posterior lens surfaces of Figure 9.

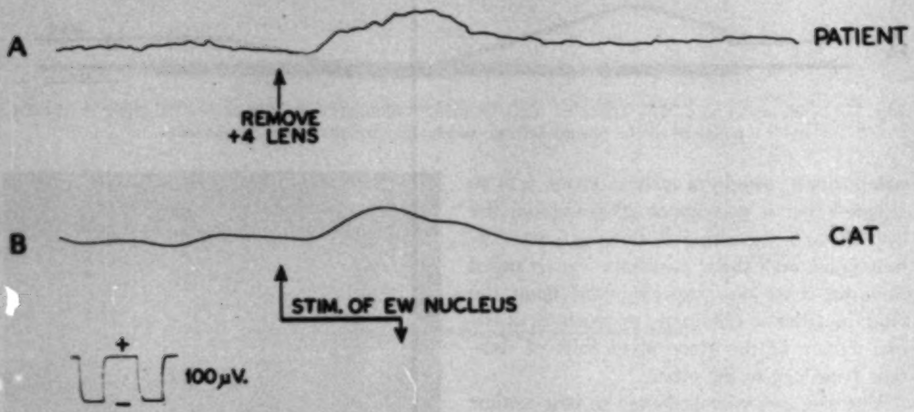

Fig. 11 (Jacobson, et al.). Curves recorded from human upon removing +4.0D. lens, and cat upon stimulation of third-nerve nucleus. Similarities are shown.

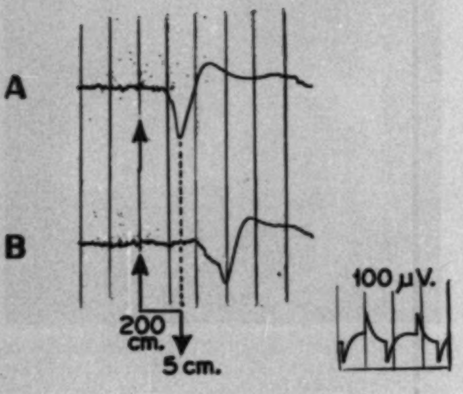

Fig. 12 (Jacobson, et al.). Increase in latency time upon repeated near focusing in human. Lower tracing after five repetitions of near focusing. Condenser-coupled amplifier.

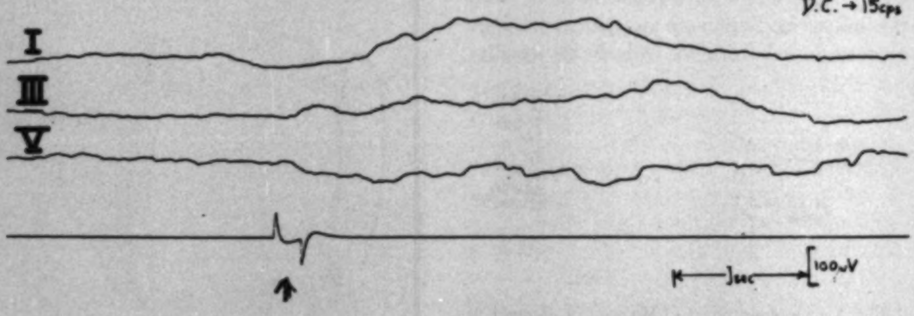

Fig. 13 (Jacobson, et al.). Diminution in human response on first, third, and fifth repeated efforts. DC recording.

scope. A push button connected to a voltage source and one of the recording channels allowed the subject to signal, according to a prearranged code, without moving the head. Every attempt was made to keep the subject as relaxed and comfortable as possible. The subject's age, sex, refractive error, and range of accommodation were determined.

In the first series of experiments nine subjects were asked to fix their attention first on one and then on the other of the superimposed targets, and the targets maneuvered so that no gross eye motions were apparent as the shift occurred. Now the distance target alone was illuminated for a short period of time, followed by illumination of the near target. The subject signalled in each instance when he was able to see the target closely.

Results. In response to a shift from far to near a long, slow potential of about 0.2 millivolts, lasting about two seconds, occurred. The potential usually preceded by a fraction of a second the subject's signal that he was able to see the near target. The potential could not be elicited by contralateral illumination. Dilation with paredrine prior to the experiment did not abolish the potential. Voluntary eye motions were carried out in response to command. The type of potential variation recorded was not of the same character, rather being a short, sharp wave.

Method 2

A second experimental apparatus was devised, in which the near target consisted of digital counting device, whose numbers were variable by a remote telephone dial. It was mounted at a distance which required the subject, in order to see the target clearly, to exert just slightly less than his maximum accommodative capacity. Provision was made for interposition, between the subject's eye and the target, of trial case lenses. A trial frame lens representing just this amount was now placed between the subject and the target, thus forcing the relaxation of his accommodation. The subject signaled when he was able to see the target clearly with the lens in place. The lens was now removed, and the subject attempted to focus upon the target.

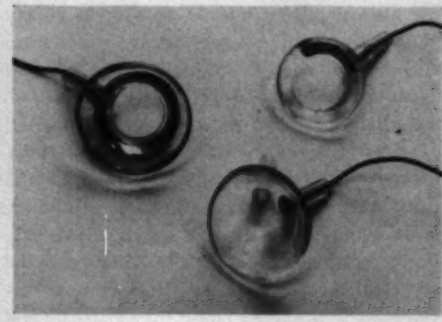

Fig. 14 (Jacobson, et al.). Types of contact lens electrodes used.

Results. In each instance a similar slow potential was recorded. The subject usually reported a delay of about one second before being able to see the near target clearly and also reported that the near vision acuity seemed to be an oscillatory phenomenon for the first few seconds, coming and going, like a picture from a projector whose focus is being adjusted. During the subjective "focusing" sensation there is usually a slow potential variation.

It was possible to fatigue the subjective ability to focus and the fatigue coincided with a diminution in amplitude of potential produced, and with an increased latency in the time of development of potential. Dilation with paredrine did not abolish the response, but cyclogyl did. Contralateral illumination produced only a very minute wave.

Discussion

Several findings are here presented. The microelectrode studies and the reaction of excised ciliary muscle to electric stimulation seem to indicate that the muscle in question functions much like other smooth muscle, although the frequency of spike discharge seems to be higher than that found in intestinal muscle.

The shape of the potential recorded from

the animal ciliary muscle area upon ciliary ganglion and third nerve nucleus stimulation and the shape of the potentials recorded from the human eye during accommodation correspond quite well to previously reported curves of the alteration in mechanical activity of the ciliary muscle and of curves of the course of objective measurement of the alteration in lens shape. This coincidence certainly makes it attractive to speculate that the potential recorded is really a measure of accommodation. We believe that a great deal more work must be done, however, to delineate the role of iris potential, lens shape, and positional variations, minor eyeball motions, electrode motions, and lid motion, before it can be assumed that these potentials are due to ciliary muscle activity. This work is now in progress.

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The technical assistance of Gidon Gestring was of great importance in this work.

References

1. Fincham, E. F.: Mechanism of accommodation. Brit. J. Ophth., **21** (Suppl.):5, 1937.
2. Glezer, V. D., and Zagorulko, L. T.: Method of physiologic investigation: Photoelectric registration of accommodation reflex. J. Physiology (Russian), **61**:830, 1955.
3. Melton, C. E., Purnell, E. W., and Brecher, G. A.: The effect of sympathetic nerve impulses on the ciliary muscle. Am. J. Ophth., **40**:155 (Dec., Pt. II) 1955.
4. Buhlbring, E.: Correlation between membrant potential, spike discharge, and tension in smooth muscle. J. Physiol., **128**:200-221, 1955.
5. Brune, H. F., and Kotowsky, H.: The excitation lead of smooth muscle of guinea pig color. Pflüg. Arch., **262**:484, 1956.
6. Schubert, G.: Human action potential of the ciliary muscle. Arch. f. Ophth., **157**:116, 1955.

CHANGES IN SPONTANEOUS AND EVOKED POTENTIALS ON THE EYES OF CATS INDUCED BY DRUGS*

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The appearance of potentials picked up from the surface of the eyes of cats is familiar to you. There are slow, low amplitude waves so long as illumination of the retina remains constant. When the intensity of illumination brightens or dims, a sharp potential change is evoked within a few msec. These are the well-known resting and evoked electroretinograms. Other potentials may appear under some special conditions, however, and these potentials are less well-known. These are the slow but high amplitude potentials that appear unevoked in dark-adapted eyes[10] (fig. 1) or following either local[12,23] or systemic administration of certain drugs[2-4,12,17,20,23] (fig. 2). These potentials are especially interesting because their origin is obscure. There are some investigators who feel that dark- or drug-induced ocular potentials are an artifact and who routinely damp them out. Other workers admit a value in observing spontaneous ocular potentials and attribute them to an extraocular vascular or corticofugal source.[10,12] In our laboratory we are concerned specifically with these potentials because they might be an unexplored indicator of retinal function.

In order to investigate the mechanism by

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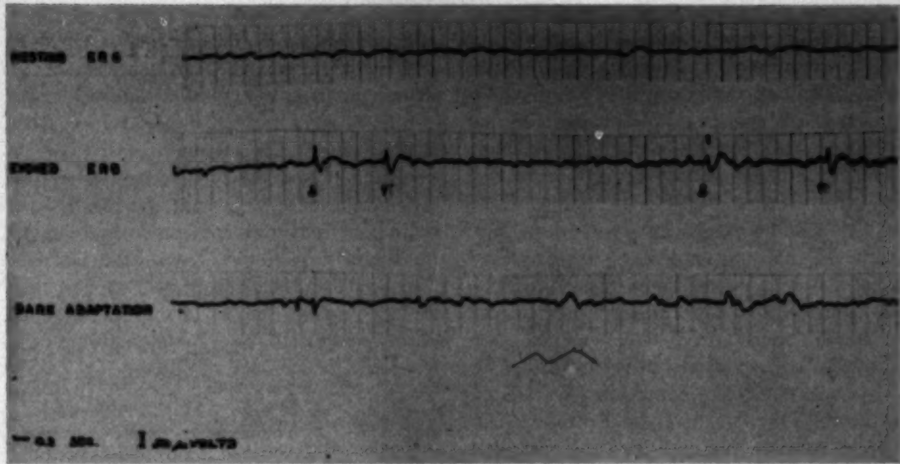

Fig. 1 (Apter). Three tracings from the eye of an anesthetized cat. Upper tracing taken with illumination at 10 foot-candles. Middle tracing taken during two flashes of a light 100 foot-candles bright. Lower tracing taken after 30 minutes in the dark, while the cat remained in the dark.

which certain drugs induce ocular potentials we have developed a reliable method for demonstrating these potentials. Using this method we have gathered data indicating that the drugs which induce potential changes in the eye have an action directly on the retina and therefore may produce ocular potentials via this structure rather than via the ciliary

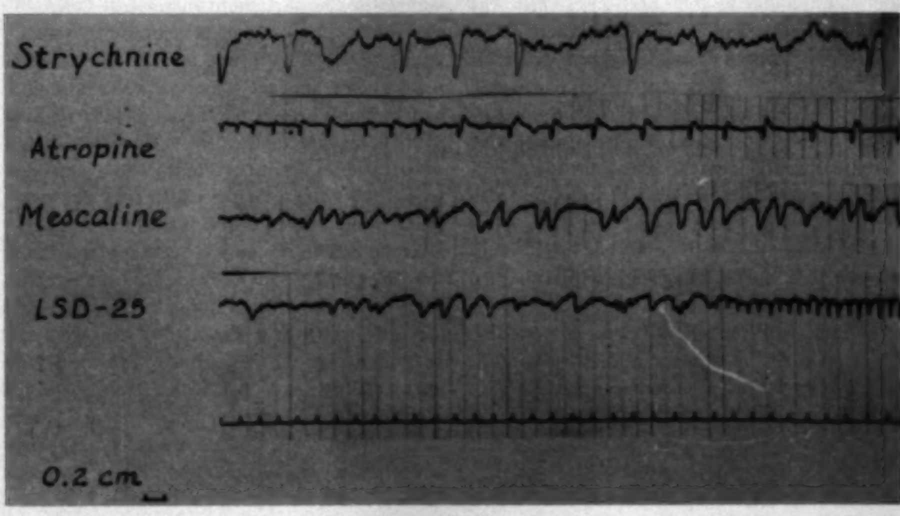

Fig. 2 (Apter). Four tracings taken from the sclera of curarized cats 15 minutes after the intraperitoneal injection of strychnine, mescaline, LSD-25, and atropine. Basal illumination constant at 20 foot candles. Lowest tracing was simultaneous electrocardiogram from the cat which received LSD because the potentials on the eye of that animal were occasionally rapid and might have been vascular in origin. However, they were much faster than the electrocardiogram.

muscle, blood vessels, or corticofugal pathways to the retina.

The present report, therefore, will be concerned with the rationale for the method used and will also present evidence for the hypothesis that strychnine, LSD, mescaline, and atropine have an action directly on the retina.

The method we use for demonstrating and studying drug-induced ocular potentials was devised after a laboratory evaluation of several widely used experimental procedures. We tested anesthetized versus nonanesthetized cats. We also tested three electrode materials and many loci for placing electrodes on the surface of the eye. Intensity of background illumination and drug dosage were considered important also. We even gave some of the drugs to human volunteers to make certain that they had the expected clinical effect of producing hallucinations. All four drugs that produced ocular potentials in cats produce spontaneous visual experiences in human subjects.[3, 4, 15, 16]

We tested the effect of anesthesia by using one nembutalized and one curarized cat simultaneously for each experiment. The ocular responses observed did not differ in the two animals. Therefore we concluded that anesthetized animals were satisfactory for a study of the ocular effects of drugs. A statistical analysis of some of the data will show this more clearly later in this presentation.

A test of five dose levels of each drug showed that for some drugs which induce ocular potentials there is a narrow range in which the drug is effective. LSD, for instance, is effective only from 25 to 50 µg./kg. and strychnine 0.1 to 0.3 mg./kg. Lower or higher doses failed to evoke potentials. Mescaline, on the other hand, was effective from 0.1 mg./kg. to lethal doses of 100 mg./kg. All these drugs were most reliable if given intraperitoneally.

We failed to evoke potentials following intravenous administration. Of course, smaller doses were used than those given intraperitoneally, but perhaps the doses were not small enough. Atropine was just as effective when instilled into the conjunctival sac as when given as 1/150 gr. subcutaneously. The dose level of each drug that was safe, easy to administer, and repeatedly successful in producing ocular potentials was chosen for the present study. Certain drugs did not evoke potentials although many doses up to lethal ones were tested. These drugs, adrenaline, ephedrine, metrazol, were used in safe per-kg. doses as judged in man to serve as control medication. See Table of drug doses.

DRUG DOSES PER KG. BODY WEIGHT

Nembutal	40 mg.	Intraperitoneal
Strychnine	0.1 mg.	Intraperitoneal
LSD-25	25 µg.	Intraperitoneal
Mescaline	10 mg.	Intraperitoneal
Metrazol	0.1 mg.	Intraperitoneal
Ephedrine	0.1 mg.	Subcutaneous
Adrenaline	0.1 mg.	Intravenous
Glucose 10 per cent	10 cc.	Intravenous
Oxygen	pure	by mask

Various electrodes[9, 10, 14, 21] have been used in electroretinography but not all are equally suited for the particular purpose of the present study. The electrodes must be nonpolarizing, immobile on the eye, and must not produce an artifact when exposed to light For example, Ag-AgCl does not polarize but it may become photically active soon after preparation. Calomel half-cell electrodes would be satisfactory because they are nonpolarizable and do not introduce a photic artifact but they cannot be immobilized on the sclera. We devised the following procedures to provide nonpolarizing immobile electrodes free from a photic artifact. Insulated platinum wire sutured to the sclera with a silk thread on a Kalt needle made an ideal electrode for these experiments. Each electrode may be placed precisely and the silk thread acts as a saline wick from the retina. Therefore, polarization was very rare and no photic artifact due to the electrodes could be seen.

Others working in this field have placed electrodes in various arrangements: cornea

to forehead, cornea to optic nerve, and so forth.[9,10,13,14,22] All placements are not equally effective and we explored several. Since we were looking for spontaneous activity, it seemed advisable to consider both electrodes as active and to place them to pick-up only from the eye. To accomplish this we sutured two electrodes to sclera overlying retina. We discovered that the electrodes never picked up drug potentials if both electrodes were over the ciliary body; nor did the potentials appear if the electrodes were aligned along an equator of the sclera over the retina (fig. 3). However, if the electrodes were aligned along a meridian of sclera overlying retina, drug-induced potentials were maximal (fig. 4). This arrangement of pick-up electrodes has been shown by Therman,[23] by Granit,[10,12] and by others to the optimal, also, for demonstration of the photically evoked retinal response.

Another factor important in the demonstration of drug potentials was the level of basal illumination. We found that five to 25 foot-candles was optimal. Illumination less than five foot-candles is accompanied by the usual occasional dark-adaptation potentials and drugs do not change this activity. In illumination of five to 25 foot-candles there are no remarkable potentials in anesthetized or curarized cats unless one of the four active drugs is given. Above 25 foot-candles not even the drugs are successful in inducing potentials although a change in intensity of light will, of course, be followed by an evoked potential wave. It appears, therefore, that light of a particular intensity is necessary for the appearance of drug-induced potentials.

Based on these explorations, the following standard procedure was adopted. Nembutalized or curarized cats were immobilized in a stereotaxic instrument with the eyes illuminated by 20 foot candles. Two insulated platinum wire electrodes were sutured to sclera overlying retina and were aligned along a scleral meridian. They were led to a Grass electro-encephalogram and to a Dumont cathode ray tube via a Grass preamplifier. A long time constant (0.2 sec.) was used to study spontaneous potentials and a shorter time constant (0.05 sec.) on the cathode ray

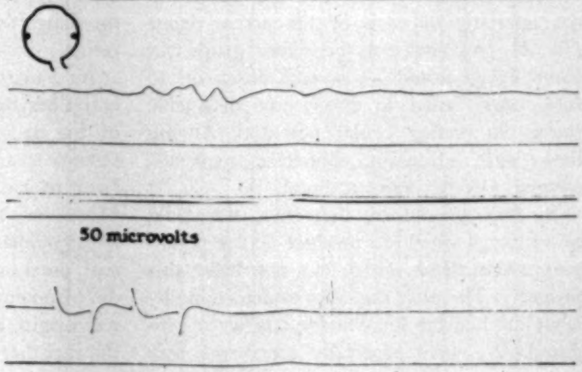

Fig. 3 (Apter). Two tracings from the sclera of a cat with electrodes aligned on a scleral equator 15 minutes after LSD was given to the cat. The optic nerves had been cut one hour before these tracings were made. Drug potentials are minimal.

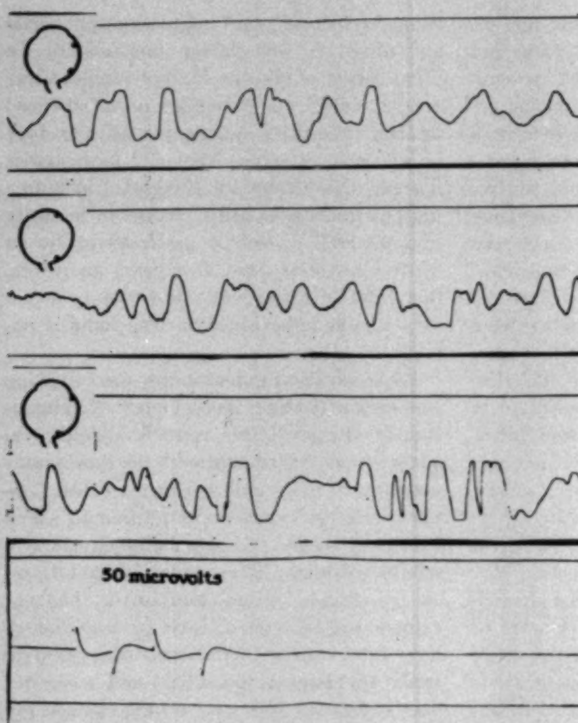

Fig. 4 (Apter). Three tracings from the sclera of the same animal as in Figure 3 under the same conditions, but the electrodes were aligned on a scleral meridian. In each instance at least one electrode was on sclera overlying retina. Drug potentials are maximal.

tube for photically evoked potentials. Ocular potentials appeared 10 minutes after LSD-25, mescaline, or strychnine, or atropine was given in the doses and via the route found to be optimal. The potentials lasted as long as two hours. These potentials may appear in several different forms and are somewhat characteristic of each of the active drugs (fig. 2). In contrast to these four drugs, six other drugs tested in several doses up to fatal ones failed to show any detectable change in resting ocular potential. Among these were adrenalin, ephedrine, metrazol, oxygen, glucose, and nembutal.

By this procedure, therefore, we could select drugs which do produce ocular potentials from those which do not have this property. The next step was to determine by what mechanism these potentials were produced. We were especially concerned with the contribution the retina might make to the drug potentials. To do this one should isolate the retina and each extraocular influence which might affect retinal function. This direct approach, however, was not feasible in our laboratory. By using several less direct approaches we were able to show that the drugs do have an action directly on the retina.

As a matter of fact, we already have several clues that the retina may be the source of the drug potentials. For one thing, the potentials are detected only on sclera overlying retina, not over cornea or ciliary body. Moreover, the electrode placement optimal for demonstration of a photically evoked retinal potential is also optimal for drug-induced potentials. Further support for a retinal origin of drug potentials comes from the fact that light is essential to their appear-

ance. This suggests that light must interact with these drugs to produce the potentials. Important also is a report we made several years ago that the drug potentials[3,4] remain for about four minutes on an eye that has had the optic nerves (fig. 4) cut behind the orbit. This finding rules out vascular or corticofugal influences as a source of ocular potentials but it does not necessarily implicate the retina.

If it is true that these drugs have a direct action on the retina, we might expect that the potentials would not appear on an eye in which the retina had been removed or damaged. This is indeed the case.

Animals with one retina destroyed were studied in the following way: Ten cats were prepared by injecting 0.1 cc. of 10-percent formalin into the vitreous chamber of one eye. One month later that eye was found to have a dilated pupil which did not respond to light, an avascular, detached retina, but a normal choriocapillaris pattern. I hope you will sympathize with me when you discover that these specimens were accidentally burned in a fixative. Other specimens are being prepared and they will be ready in time for publication. Our usual experiment was carried out on these cats with two electrodes on the normal and two on the injured eye. Drugs induced changes only in the activity of the normal eyes. There was no evoked potential on the injured eye but there were low amplitude, slow spontaneous potentials. These spontaneous potentials were not changed by the drugs which were active in the normal eye. This suggested that the drugs produce their ocular effects only in the presence of an intact retina (fig. 5).

If the drug potentials do indeed arise as a result of retinal discharges, we might expect that the drugs would also induce other evidences of altered retinal function. We already have some evidence that this is the case. In previous publications we reported that LSD-25 and strychnine increased the negative a-wave of the electroretinogram and that small wavelets were superimposed on part of the b-wave[2-4] (fig. 6). Both these changes are characteristic of the electroretinogram to a light of high intensity[5,9,10,14,21] yet the photic stimulus was only 150 lumens in a light-adapted eye. These findings suggest that the drugs do alter retinal function. We

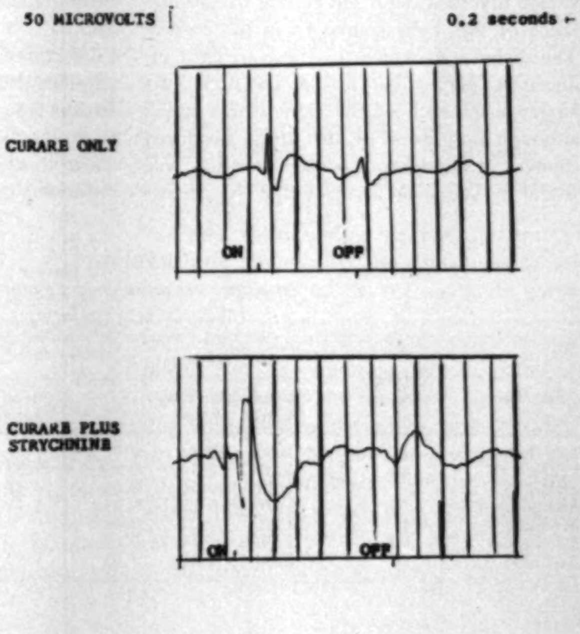

Fig. 5 (Apter). Photomicrograph of a section of the eye of cats which had 0.1 cc. of 10-percent formalin injected into the vitreous chamber one month previously. The retina is detached and atrophic. The choroid and sclera are intact. This eye showed no drug potentials.

TABLE 1

CHANGES IN THRESHOLDS

(centimeters squared between source of light and cat's cornea)

Test Drug	Number of Cats	Nembutal Mean Change	Nembutal Standard Error	Nembutal P	Curare Mean Change	Curare Standard Error	Curare P	P(N vs C)
Strychnine	12	2.08	0.49	0.01	2.50	0.51	0.01	0.05
LSD-25 IP	12	2.08	0.46	0.01	2.00	0.45	0.01	0.05
LSD-25 IV	7	1.57	0.39	0.01	2.42	0.54	0.01	0.05
Ephedrine	8	1.80	0.33	0.01	1.62	0.36	0.01	0.05
Adrenaline	10	1.70	0.29	0.01	1.70	0.34	0.01	0.05
Metrazol	11	1.64	0.33	0.01				
Atropine	26	8.42	0.98	0.01	6.81	1.12	0.01	0.05
Mescaline	7	1.87	0.40	0.01	1.99	0.53	0.01	0.05

might expect to get further information about the effect of the drugs on the retina from threshold studies and from the study of the retinal response to a flickering light. This approach would be especially fruitful since threshold changes would indicate a change in rod-function while a rise in fusion frequency to an intense light would indicate improved cone function.[6, 7, 10, 18, 19]

In threshold studies, the source of light was a screen illuminated by a strobolux and strobotac unit. The intensity of the flash of light on the cornea of dark-adapted cats varied inversely with the square of the distance of this light source from the cornea. The distance at which this flash of light induced a perceptible action potential was longer after each of the eight drugs tested although only four of the drugs produced changes in spontaneous ocular potentials. We found by statistical analysis that this reduction in threshold was of the same order of magnitude for each drug except atropine.

The drugs had a similar effect on critical fusion frequency. Fusion frequency was taken as the frequency of an interrupted photic stimulus at which photically evoked potentials in the electroretinogram disappear. This frequency at which fusion occurred increased after each of the eight drugs tested. The increase was of the same magnitude after each drug except atropine.

We analyzed statistically the findings on thresholds and flicker fusion frequency in nembutalized and curarized cats (tables 1 and 2).

A paired comparison test showed that a significant drop in threshold and increase in fusion frequency followed each drug tested. A group comparison test, however, failed to show that nembutalized cats differed from curarized cats in these particular ocular re-

TABLE 2

CHANGES IN FLICKER FUSION FREQUENCY .

(flashes of light per second)

Test Drug	Number of Cats	Nembutal Mean Change	Nembutal Standard Error	Nembutal P	Curare Mean Change	Curare Standard Error	Curare P	P(N vs C)
Strychnine	12	1.66	0.45	0.01	1.58	0.48	0.01	0.05
LSD-25 IP	12	1.66	0.76	0.01	1.83	0.48	0.01	0.05
LSD-25 IV	7	2.14	0.56	0.01	2.43	0.46	0.01	0.05
Mescaline	7	2.57	0.65	0.01	3.14	1.1	0.01	0.05
Metrazol	11	1.81	0.32	0.01				
Adrenaline	10	2.20	0.69	0.01				
Atropine	26	5.50	1.12	0.01	5.50	0.84	0.01	0.05

sponses. A covariance analysis of all eight drugs was compared with a covariance analysis of all the drugs excluding atropine. By this method we failed to show that strychnine, mescaline, LSD-25, ephedrine, adrenalin, or metrazol differed in their effect on retinal threshold or on critical fusion frequency.

On the other hand atropine has an effect significantly greater than the other drugs. The greater effects of atropine may be attributed to the pupillodilatation induced by atropine. A slight increase in pupil diameter alters threshold and flicker fusion levels by increasing the intensity of light which reaches the retina.

It may be that a change in pupil size sufficient to mask a true retinal effect was responsible for our failure to show a difference between active and control drugs. This certainly is a possible explanation since a slight change in the amount of light incident on the retina would result in measurable changes in the evoked electroretinogram in threshold or flicker fusion studies.[1,5,18,19,24] Therefore, while we have not shown that potential-evoking drugs differ from control drugs, this avenue of study will not be abandoned. A meticulous control of the amount of light reaching the retina on which these drugs may be acting may yield more specific information.

Another investigation, on the other hand, did yield conclusive data indicating that the active drugs alter retinal function. In this study we measured the duration of time during which an electroretinogram may be evoked by a photic stimulus after the eye was removed from the orbit. We called this time the persistence time. It was investigated in the following way. Two electrodes were placed on both eyes of 74 cats. One eye of 54 cats was dark-adapted for one hour. Then the eye was enucleated with the electrodes in situ on the sclera. Following section of the optic nerve, a photic stimulus was flashed through the pupil and the photic response was noted. The time from section of the optic nerve to disappearance of the photic response was taken as the persistence time (table 3). This is four minutes in a dark-adapted eye.

TABLE 3
FINDINGS AFTER ENUCLEATION

Drug	Number of Cats	Persistence Time (Min.)	C. V. in %
Nembutal (dark-adapted)	64	4	14%
Nembutal (light-adapted)	33	2	10%
LSD-25	30	17	15%
Mescaline	21	17	11%
Atropine	14	12	7.2%
Strychnine	32	32	12%
Metrazol	12	4	16%
Ephedrine	10	4	14%
Adrenalin	10	4	12.5%
Glucose	3	4	
Oxygen	4	4	

Thirty-two cats were light-adapted to five foot-candles before enucleation and their persistence time was two minutes. The other eye of each of these 74 cats was treated as follows: The cats were given a test drug (LSD-25, strychnine, atropine, or mescaline) or a control drug (glucose, oxygen, metrazol, ephedrine, or adrenalin) intraperitoneally. Fifteen minutes after this injection (the four active drugs having produced potentials in the light-adapted eyes) the second eye was enucleated and treated like the first eye. The persistence time was 17 minutes after LSD, 12 minutes after atropine, 17 minutes after mescaline, and 32 minutes after strychnine. None of the drugs which do not induce ocular potentials changed the persistence time.

From these studies we find that proper methods can readily demonstrate ocular potentials evoked by drugs. Since the potentials are seen only over an intact retina and only when there is a certain amount of light in the eye, it appears that the drug potentials may originate in the retina. Moreover, since the drugs prolong the ability of the retina of an isolated eye to show an evoked potential after photic stimulation, it appears that the drugs affect the retina in a specific way in addition to inducing ocular potentials.

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References

1. Adrian, E. D., and Matthews, R.: The action of light on the eye: Part III. The interaction of retinal neurones. J. Physiol., **65**:273-298, 1928.

2. Apter, J. T.: Prolonged survival of the retina of an isolated eye induced by drugs strychnine, mescaline, LSD-25, and atropine. Arch. Ophth., **59**:722-730, 1958.

3. Apter, J. T., and Pfeiffer, C. C.: Effect of hallucinogenic drugs on the electroretinogram. Am. J. Ophth., **42**:206-210 (Nov. Pt. II) 1956.

4. ———: The effect of the hallucinogenic drugs LSD-25 and mescaline on the electroretinogram. Ann. New York Acad. Sci., **66**:508-513, 1957.

5. Berger, C.: Area of retinal image and flicker fusion frequency. Acta physiol. Scand., **28**:224-233, 1953.

6. Dodt, E., and Enroth, C.: Retinal flicker response in cat. Acta physiol. Scand., **30**:375-390, 1953.

7. Dodt, E., and Wirth, A.: Differentiation between rods and cones by flicker retinography in pigeon and guinea pig. Acta physiol. Scand., **30**:80-89, 1953.

8. Dusser de Barenne, J. G.: Physiological delimitation of neurons in the central nervous system. Am. J. Physiol., **127**:620-628, 1939.

9. Granit, R.: Isolation of components in the retinal action potential of the decerebrate dark-adapted cat. J. Physiol., **76**:1, 1932.

10. ———: Receptors and sensory perception, New Haven, Yale Univ. Press, 1955, p. 366.

11. Granit, R., and Munsterhjelm, A.: The electrical responses of dark-adapted frog's eyes to monochromatic stimuli. J. Physiol., **88**:436-458, 1937.

12. Granit, R., and Therman, P. O.: Excitation and inhibition in the retina and the optic nerve. J. Physiol., **83**:358-381, 1935.

13. Gunther, R.: The spectral sensitivity of dark-adapted cats. J. Physiol., **118**:395-404, 1952.

14. Hartline, H. K.: The electrical response to illumination of the eye in intact animals, including the human subject and in decerebrate preparations. Am. J. Physiol., **73**:600-612, 1925.

15. Heinbecker, P., and Bartley, S. H.: Manner of strychnine action on the nervous system. Amer. J. Physiol., **125**:172-187, 1939.

16. Klüver, H.: Mechanisms of hallucinations. Chapter X in Studies in Personality. New York, McGraw-Hill, 1942, pp. 175-207.

17. Marshall, W. H.: Personal communication.

18. Müller-Limmroth, H. W., and Andreé, G.: Untersuchungen Zwo Differenz konstruktion des Elektroretinogramms des Froschauges. Ztschr. Biol., **103**:324-328, 1953.

19. ———: Das Electro-retinogram bei Flimmerbelichtung. Pflüg. Arch. Physiol., **257**:216-253, 1953.

20. Noell, W.: Studies on the electrophysiology and metabolism of the retina. USAF School of Aviation Medicine Project No. 21-1201-0004. Report No. 1, 1953.

21. Ottoson, D., and Svaetichin, G.: Electrophysiological investigations of the frog retina. Cold Spr. Harb. Symp. Quart. Biol., **17**:165-173, 1952.

22. Rovetta, P.: Effect of mescaline and LSD on evoked responses especially in the optic system of the cat. Electroencephalog. & Clin. Neurophysiol., **8**:15-42, 1956.

23. Therman, P. O.: The neurophysiology of the retina in the light of chemical methods of modifying its excitability. Acta. Soc. Sci. Fenn II, No. 1, Helsingfors., 1938.

24. Wirth, A., and Zetterstrom, B.: The effect of area and intensity on size and shape of the ERG: The exclusion of stray light effect. Brit. J. Ophth., **38**:257-265, 1954.

Discussion*

Dr. Albert M. Potts (Cleveland): As you observed, despite the fact that all these papers have in common the measurement of some electric response in the eye, they are nevertheless markedly dissimilar. Since there are a number of individuals in the audience who have specific interests in one or another of these fields, I am going to confine myself to a few general remarks.

As someone who has been interested in ocular electrophysiology for some time, these papers say to me as do my own efforts: "How presumptuous you people are to do your experiments and hope to find the answer to this extremely complex, exquisite machine, with your instruments and oscilloscopes that take up a whole room with electronic gear, and that can't approach in elegance the tiny, submicroscopic aspects of the organ that you are trying to elucidate."

The paper by Dr. Fuortes simply extends the quiet smile of Dr. Hartline when anyone talks to him about doing mammalian electrophysiology. Here are some very elegant parameters on a simpler invertebrate model that is a true photoreceptor; (but which recently has been found to have more

* Included in this discussion are the preceding four papers—by Fuortes, by Granit and Marg, by Jacobson, et al., and by Apter.

complicated aspects) and which compared to the mammalian eye is an easier experimental preparation.

This does not in any way minimize the difficulties of inserting microelectrodes into single ommatidia of the Limulus eye, and it does not minimize the very likely prospect that this type of polarization potential will be found across the eyes of warm-blooded animals when we are clever enough to be able to do the experiments.

In Dr. Marg's experimentation, the fact that different conduction velocities indicated different sizes of nerve fibers confirms the things that we know about conduction velocity and fiber size in general. But again when we start talking about extrapolation of these very interesting findings to visual function, a single impulse either of electricity or of light is an extremely simplified analog of the visual process. The visual process is something that during actual function varies very slightly indeed as far as light levels are concerned and as far as these levels on various areas of the retina are concerned. To extrapolate difference in conduction time to this complex natural situation may be possible but only if one considers integration over a long period of a whole series of impulses that make up the continuous visual picture.

Dr. Jacobson's paper, which was not on visual function at all, again indicates (as Dr. Jacobson himself mentioned) how extremely difficult it is to get answers from an organ like the ciliary body, which has a delicate blood supply and which is protected by the sclera from most of our assaults. I would like to ask Dr. Jacobson one question which occurs about a control: What happens to these potentials in the various circumstances when cycloplegics are used in the experiment?

Dr. Apter confuses me entirely. She undoubtedly has potentials, and she can get them with substances like LSD or mescaline, which in some humans give visual hallucinations at particular levels. She gets such potentials in the eye at greatly varying drug dosages, and she gets them also by dropping atropine in the eye. Here again, these things are real, but when we try to convert them to some very pertinent conclusion about the visual process, nature frustrates us once more.

Now I would like to turn the subject over to general discussion, because a number of persons here are particularly interested in various aspects of the papers that have been presented.

Julia T. Apter (Chicago): I am glad to be the first to give a rave notice to Dr. Fuortes for his wonderful study. If we could all model our studies along the lines he has followed, we would get a whole lot farther ahead. I think it is possible that by using Dr. Fuortes' results and methods we may be able to get a lot farther using electric stimulation, a method that has, up until now, had many, many limitations.

I am happy that the unique ability of finding these potentials has been imputed to me but I must decline, because I only found lysergic acid and the mescaline potentials. Therman and Granit found the strychnine and Marshall found the atropine potentials.

Although lysergic acid and mescaline were assumed to be hallucinatory drugs acting at a high level in the nervous system, there was no really good reason for that assumption. The drugs, as you may know, have been called psychomimetic because they produce visual hallucinations in normal human subjects. They also induce other changes in personality. Once having been so labeled, neurophysiologists attempted to find where in the central nervous system they acted.

Do you know that they studied the concentration of that drug in every organ of the body except the eye? They traced it, after they labeled it, in every organ of the body except the eye, although in 1942 Klüver was able to reproduce mescaline-like hallucinations in human subjects by certain kinds of visual stimulation.

You should know that the visual hallucinations that all of these drugs produce are not formed hallucinations; they are actually the very same kind of experience that you get when you rub your eyes. It is a phosphorent light with a grating superimposed on everything. The grain of wood seems to come alive. Klüver felt (and I feel, too) that the characteristics of these hallucinations are best explained on the basis of intraocular structures. That is why I looked in the eye and found these spontaneous potentials, and found that all of the drugs have other effects on the retina also.

I am hoping that Dr. Alpern, who is going to talk about flicker fusion studies in humans following autonomic drugs, will find that they have some effect on flicker fusion in humans, because I find that these drugs increase the flicker fusion frequency at the retinal level and also lower thresholds in the retina.

I would like to ask Dr. Jacobson a question and make one suggestion that he may find useful. First, the suggestion:

I have worked with the ciliary ganglion and find that it is not difficult to get at if you hold the jaw open and lower the ramus of the mandible from the lateral wall of the orbit. The lateral wall of the orbit in a cat is mostly muscle. The ramus of the mandible forms part of the lateral wall. If the jaw is opened that will not get in the way. We find it rather easy to reach, and I believe there are some people at Iowa State University who are reaching it for stimulation also. There are long discussions in the literature about how stimulation of the ciliary ganglion is superior to the third-nerve stimulation for demonstrating the parasympathetic effects.

I would like to know the rationale for the 200-per-second stimulation that you used, Dr. Jacobson. Electrical stimulation for inducing motor activity varies from 10 per second up to 300 per second, and I wonder if there was a particular reason why you chose 200 per second.

Dr. Gerhard A. Brecher (Atlanta, Georgia): Dr. Potts raised a question (and Dr. Jacobson can answer this) as to whether there is anything known

about the effect of cycloplegics on the ciliary muscle in these recordings.

Dr. Jacobson pointed out that Schubert was the first who recorded ciliary muscle potentials in man. I don't recall the details of Dr. Schubert's paper, but Schubert told me that he used cycloplegics and did not obtain any potentials. I do not know whether Dr. Jacobson did the same, or whether it was a passage in Schubert's paper to which he referred.

Dr. Jacobson's work is very interesting in so far as he tried to correlate Schubert's in toto recording of ciliary muscle potentials in man with the isolated ciliary potentials in the cat.

Dr. Apter mentioned the ciliary ganglion stimulation. It is superior to the third nerve stimulation; and, although it is not an easy dissection to get to the ciliary ganglion, it is the best.

The next question is, which role does the iris muscle play here? Schubert prevented the action of the iris muscle by a suitable optical arrangement.

Concerning Dr. Apter's work, as interesting as it is, I fail to see why she has not taken the opposite approach. Why she didn't start her experiments in the most controlled situation, that is, with the recording from the retina itself.

For example, from the frog or cat this would provide an experimentally clear-cut situation which might have brought an easier and more direct solution to the problem.

What about the mescaline-induced hallucinations of man whose optic nerves were destroyed? Are there not some descriptions in the old literature stating that such people also had visual hallucinations? I did not read those descriptions, but only heard of them. I would be interested if you have further information on this point.

Dr. Mathew Alpern (Ann Arbor, Michigan): First of all, I would like to second Dr. Apter's remarks about the beautiful experiments of Dr. Fuortes. I would like to ask him if he has extended his observations to analyzing the problem of the interaction of adjacent elements in the compound eye of the Limulus. Specifically, what effect does polarization of a given ommatidium have on the photic response not of the same ommatidium but of one of the adjacent ommatidia?

Dr. Goldsmith, Dr. Ellen, and I have made a good many observations on the human accommodation electric changes. I agree with Dr. Jacobson that we need a considerably greater amount of purely physiologic experiments before we can conclude that these are in fact ciliary muscle potentials.

I think the major problem is to find out how it is possible to obtain these very slow DC changes associated with accommodation by integration, or whatever it is, of the high frequency potentials that were recorded with the microelectrodes from the isolated muscle.

Dr. Apter asked about the effect of autonomic drugs upon human flicker discrimination and there is little doubt that such effects do occur. On the other hand a considerably greater amount of purely physiologic experiments need to be carried out before the relation between these psychophysical experiments and her work will become clear.

The question I would like to ask Dr. Apter pertains to work on the photo-induced electrophysiologic changes which accompany mescaline and lysergic acid. The question is this:

Are these effects real or are they related to some artifact of the polarization of the electrode? There seems to be a controversy in which some authorities feel that these changes are physiologic while others think they are due to some artifacts of the electrode. I would like to have this matter cleared up.

Dr. Fuortes (Bethesda, Maryland): May I say first of all that I am very grateful for the generous comments to my paper.

In answer to Dr. Alpern's question, I have not stimulated electrically one eccentric cell while recording from an adjacent unit. The purpose of the research I have reported was simply to determine some of the elementary properties of eccentric cells. Only very recently I had an opportunity to confirm Hartline's findings on lateral inhibition (Hartline, H. K., and Ratliff, F.: J. Gen. Physiol., **40**:357-367, 1957; Hartline, H. K., Wagner, H. G., and Ratliff, F.: J. Gen. Physiol., **39**:651-673, 1956) but I did not study this phenomenon systematically. It will certainly be very interesting to analyze in detail the changes occurring during inhibition and I plan to do this in the future.

Dr. Elwin Marg (San Francisco): Just two short comments. First, in relation to a question by Dr. Apter concerning the frequency of accommodative stimulus:

I would like to mention a paper that appeared several years ago by Marg, Greeves, and Wendt in the *American Journal of Optometry*, on the response of accommodation to electric stimulation of the ciliary ganglion in the cat. This work showed that with adequate voltage, 30 to 40 cycles per second or higher, there would be a maximal accommodative response.

I know I can say for myself, and probably for my colleagues who presented papers here with me, that we do not expect to explain all the multiple intricate facets of the visual systems in our electrophysiologic research.

Dr. Jerry H. Jacobson (New York): In reply to Dr. Brecher, whose work was of extreme value to us in adding another portion to this story that we are trying to put together, our findings were in agreement with those of Schubert in the human. Atropine and Cyclogyl abolished the responses. Paredrine did not.

We felt this was also in keeping with some work that Dodt did on the iris potential. He found that if a specific type of contact lens was used, he could not get an iris potential, whereas if an electroretinogram type lens were used, he could. We seemed to substantiate that.

Concerning Dr. Apter's question about the frequency—as Dr. Marg remarked, we just picked this because it seemed to give us the best results. We

tried a number of different parameters of stimulation, and picked the one that seemed to give us the best response.

Finally, to Dr. Alpern—we said not "ciliary" activity but "accommodative" activity. To Dr. Potts, whose humility I completely share, may I say that I feel that when we are trying to learn something about as complex a mechanism as this one—and this includes Dr. Fuortes', in my opinion, which is certainly not a simple area—we are something like a child who attacks a television set with a screwdriver. We may come up with something that is interesting, but we have a long way to go before we know exactly what it is all about.

Dr. Julia T. Apter (Closing): The possibilities that my findings might be due to my electrodes—well, we found it with every known electrode, and the electrodes that showed spontaneous potentials also showed photically evoked potentials in the same way other electrodes do.

It is true that we had silver chloride electrodes at first. At that time we were not using the photic stimulus. In another laboratory using the same methods, the same potentials appeared and the electrode experts were all standing around, while the potentials appeared. Whether it was agreed or not that they came from the retina, the potentials did appear and that they were not artifacts was certain even then.

I would hope that if you would read the entire paper you would see that since we find so many other changes in the retina, certainly even if the potentials were an artifact the drugs still have an effect on the retina.

To Dr. Brecher: I am not aware of any studies in which hallucinations appeared in patients who had optic nerves cut. I do know of several studies in such patients and the patients did not get hallucinations either with mescaline or lysergic acid. As a matter of fact, one patient who had one eye removed got visual hallucinations with lysergic acid. When the second eye had to be removed and lysergic acid was given, he did not get hallucinations.

There is one case in the literature of a patient with his cataracts removed. When he was not wearing his refractive corrections he did not get hallucinations with LSD, but when he was wearing his corrections he did get hallucinations. I don't consider this very good proof but I do think the drugs have an effect on the retina.

MECHANISM OF CORNEAL DESTRUCTION BY PSEUDOMONAS PROTEASES*

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Introduction

Infections of the eye caused by Pseudomonas aeruginosa are uncommon but when they do occur they are of prime importance because of the severity of the disease and the difficulty in treatment. For these reasons we have focused our attention on this particular infection with the idea that results from studies of such a system may be useful in understanding similar type infections in the eye caused by organisms other than Pseudomonas aeruginosa. Our approach has been to study the mechanism by which the invading organism can initiate and cause tissue damage. In a paper presented at the fall meeting of the Southern Section of the Association for Research in Ophthalmology, we reported that a proteolytic enzyme elaborated by Pseudomonas aeruginosa could cause severe ocular damage when inoculated intracorneally.[1] We were able to partially purify the responsible protease which caused very extensive corneal destruction after intracorneal inoculation into rabbit eyes. Preliminary studies regarding the kinetics of the Pseudomonas protease were reported at the 1958 meeting of the Federated Societies of Experimental Biology.[2] It was shown that the optimum pH for activity was around pH 7.4 and that the temperature optimum for enzyme activity was high. Evidence was also presented which strongly suggested that a metal cofactor was important for activity of the enzyme.

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Further studies concerning this protease and its possible relationship to ocular damage will be reported in this paper.

Methods

Protease. The protease enzyme, as used in the experiments to be described, was obtained in the following manner. A strain of Pseudomonas aeruginosa, proven to be virulent for the eye,[2] was grown on a Difco nutrient agar medium for 48 hours at 37°C. The cells were harvested by washing the growth from the surface of the medium with distilled water. To remove the cells, this material was subjected to centrifugation, 10,000 X g for 30 minutes. It was found that the protease resided in the supernatant and could be precipitated by adding solid ammonium sulfate to a final concentration of 3.3 M; then the solution was allowed to remain overnight in the refrigerator, the precipitate then collected and dissolved in cold 0.15 M NaCl. This solution was dialyzed against several changes of cold distilled water at a temperature of approximately 5° to 8°C. The material was reprecipitated following the same procedure as described above. After dialysis the enzyme solution was concentrated by means of the lyophilization procedure. The resulting preparation is the one employed in the following experiments.

Animal studies. In vivo studies were carried out by inoculating preparations intracorneally into the eye of healthy rabbits.

Proteolytic assays. Two different methods were found useful for the in vitro assay of proteolytic activity. The diazoprotein method has been reported in earlier publications.[1,2] The method followed for assay of proteolytic activity against corneal proteins and collagen was a modification of the one reported by Kunitz.[3] Our reaction mixture consisted of corneal protein, buffer (Tris [hydroxymethyl] aminomethane, 0.2 M, pH 7.5), and enzyme. Protein substrates other than corneal protein were tested by this method. One ml. samples were removed at indicated time intervals and undegraded protein precipitated with three ml. of five percent trichloroacetic acid. The optical density of the filtrate was measured at 280 mμ.

Corneal proteins. Frozen rabbit corneas, collected in the same manner as recorded by Guidry, et al.,[4] were used for preparation of the corneal fractions. The thawed corneas were suspended in cold 0.15 M NaCl and minced in a Virtis "45" homogenizer. The tissue fluid ratio for mincing was one gram weight of tissue to two ml. 0.15 M NaCl. After homogenization, the soluble portion was separated from the insoluble fraction by centrifugation at 4°C. and 30,000 X g. The soluble portion, the clear supernatant fluid obtained after centrifugation, was designated corneal fraction II. The corneal fraction I was a one gram wet centrifugation sediment which had been resuspended in 10 ml. 0.15 M NaCl.

Antiserum. Antiserum was prepared against the Pseudomonas protease by means of routine immunization procedures as outlined in Boyd's Immunology.[5] The protease was inoculated intravenously into a rabbit every third day for two weeks followed by weekly inoculations for the two successive weeks. The rabbit was bled via a cardiac puncture and the serum collected after adequate clotting was obtained. The antibody level of the serum was determined in relation to its ability to inhibit proteolytic activity of the protease enzyme. The enzyme was preincubated with dilutions of the antiserum for 30 minutes to one hour after which time the proteolytic activity was determined using diazocasein as the enzyme substrate.

Agar-gel diffusion procedures. The method followed is one reported by Feinberg.[6] A one-percent Difco agar saline was prepared including sodium azide as the preservative. Agar plates were poured to contain 30 to 35-ml. agar suspension. After the agar had been allowed to gel a five-mm. cork borer was used to punch wells into the agar. There were five wells per plate arranged so that one was in the center and four were equally

TABLE 1

PSEUDOMONAS PROTEASE ACTIVITY AGAINST DIAZOCASEIN

Enzyme	*Proteolytic Activity**	*Protein Content*
Protease	16,600	0.9 mg.

* Proteolytic activity expressed as μg of diazocasein degraded in six minutes at 37° C.

spaced one cm. from the outer rim of the center well. The protease enzyme was delivered into the center well and varying dilutions of the sera added to the outer wells. The plates were incubated at 37°C. over saline in a closed container.

RESULTS

Effect of Pseudomonas protease on corneal proteins. The protease tested in these studies was prepared in the same manner as indicated in the methods section. Proteolytic activity of the protease enzyme was determined when diazocasein was the substrate. As is shown in Table 1, the enzyme was very active on diazocasein. When the enzyme is inoculated intracorneally into a rabbit's eye, extensive tissue damage results with perforation of the cornea (fig. 1). The activity of the enzyme was determined when the corneal protein fractions, described in the methods section, were tested as substrates. Incubation mixtures included 7.5-ml. corneal protein substrate, 5.0-ml. Tris buffer (0.2 M, pH 7.5), and 1.5-ml. enzyme added at zero time. The reaction mixture was incubated at 37°C. and 1.0-ml. samples removed at the indicated time intervals. It may be seen in Figure 2 that the enzyme is active against the corneal fraction. During the incubation reaction, visible clearing was observed in the mixture, probably due to solubilization of the previously insoluble material.

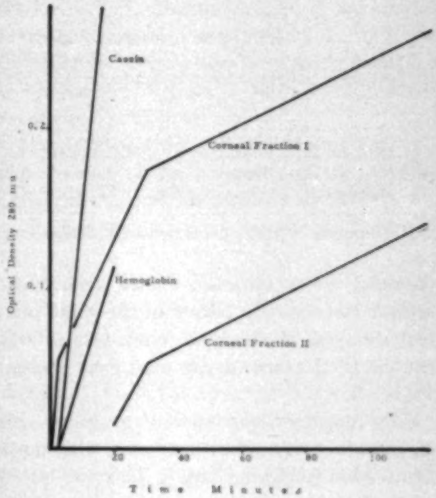

Fig. 2 (Fisher and Allen). A comparison of the Pseudomonas protease proteolytic activity against various protein substrates.

The enzyme was also incubated with corneal fraction II. Again the enzyme readily degraded this protein as it had done for fraction I, Figure 2. When one compares the activity of the enzyme against the corneal fractions, it is obvious that both fractions were readily degraded but more activity was

Fig. 1 (Fisher and Allen). Photograph taken 24 hours following intracorneal inoculation of Pseudomonas protease preparation into the eye of a rabbit.

TABLE 2

PSEUDOMONAS PROTEASE COLLAGENASE ACTIVITY TOWARD TENDON COLLAGEN

Activity Expressed as Optical Density at 280 mμ		
Protease Alone	Collagen Alone	Protease Collagen
0*	0.017*	0.265*

* Incubation time was one hour at 45°C.

TABLE 3

EFFECT OF THE SPECIFIC ANTISERUM AGAINST PROTEOLYTIC ACTIVITY OF THE PSEUDOMONAS PROTEASE

	Antiserum Dilution								
	No Serum	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
P.A.*	1990	40	30	60	80	110	190	330	720
% Inhibition	0	98	98.5	97	96	94.5	90.5	83.5	63.4

* Proteolytic Activity expressed as μg of diazocasein degraded in six minutes at 37°C.

obtained against fraction I than against the second fraction. By means of the same proteolytic assay method, the enzyme was also shown to degrade casein and hemoglobin, Figure 2.

The enzyme was shown to have collagenase activity when incubated with purified tendon collagen, Table 2. This was tested by methods described previously where liberated materials having a high absorption at 280 mμ were measured.

Antiserum studies. Antiserum was prepared against the Pseudomonas protease by procedures outlined previously in the methods section. Preliminary studies involving antiserum effects indicate that antibodies were produced against the enzyme, Table 3. In this experiment, the enzyme was preincubated with varying dilutions of antiserum, the preincubation time being 30 minutes. Proteolytic activity of the enzyme was then determined using diazocasein as the substrate. There is inhibition in all the antiserum concentrations tested even though the concentration of enzyme in all instances was rather high.

It was thought that a more adequately controlled experiment should be performed since normal serum might also contain substances inhibitory to the protease. Aliquots of the enzyme were preincubated, for one hour, with both normal serum and antiserum and proteolytic activity of the enzyme was then assayed against diazocasein. It was found that normal rabbit serum also exhibited inhibitory properties for the protease, Table 4. However, the inhibitory action of normal serum was not as great as that observed when antiserum was tested; this suggests that the antiserum probably contains antibodies against the protease which inactivate the enzyme.

Experiments were performed using the agar gel diffusion technique. Both normal serum and specific antiserum were tested as undiluted aliquots and in dilutions of 1:2, 1:4, and 1:8. Single bands of precipitation were obtained with all the dilutions of antiserum in the presence of the given protease antigen while none of the normal serum dilutions gave bands of precipitation because specific antibodies were not present in this serum. Work is in progress to determine possible protective action of the antiserum

TABLE 4

A COMPARISON OF THE EFFECT OF NORMAL SERUM AND ANTISERUM AGAINST PROTEOLYTIC ACTIVITY OF THE PSEUDOMONAS PROTEASE

		Serum Dilutions											
		No Serum	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Normal Serum	P.A.*	1250	70	90	100	130	230	230	—	640	880	960	960
	% Inhibition	0	94.6	92.8	92	90	81.5	81.5	—	49	30	16	16
Anti-Serum	P.A.*	1250	20	20	15	15	20	25	60	65	150	400	640
	% Inhibition	0	98.4	98.4	98.8	98.8	98.4	98	95.2	94.8	88	68	49

* Proteolytic activity expressed as μg. of diazocasein degraded in six minutes at 37°C.

against the protease as detected under in vivo conditions.

Discussion

The protease isolated from Pseudomonas aeruginosa has been found to cause considerable corneal damage in rabbit eyes. The enzyme has also been shown to readily degrade casein, hemoglobin, tendon collagen, and two corneal protein fractions as measured by the proteolytic assay procedure when these substrates were tested in vitro. It is of interest to note that corneal fraction I is related to insoluble materials, or materials of low solubility, as found in the cornea. This fraction therefore probably contains the corneal collagen which is concerned with structure of the cornea. In vitro experiments clearly demonstrated the proteolytic activity of the protease against this collagen-like material. The described process may be related to gross destruction of cornea as observed when the protease is inoculated intracorneally into the eye of a rabbit. The protease enzyme does have collagenase activity as tested against tendon collagen. Apparently the protease destroys tissue structure of the eye resulting in perforation of the cornea which could actually be the result of solubilization of structural proteins. The protease is also active against soluble corneal proteins as was demonstrated by its action on corneal fraction II.

The studies involving protease-antiserum production indicate that antibodies are formed in response to the enzyme stimulus. Antibodies apparently combine with the antigen or enzyme to inhibit proteolytic activity of the protease. Inhibitory action by normal serum was also observed. This could possible be explained as due to several different factors. First the rabbit bled for normal serum may have had previous exposures to Pseudomonas aeruginosa so that antibodies to the agent were already present in the blood. If this inhibitory action of normal serum is the result of antibody activity against the enzyme antigen, then the agar gel diffusion titration technique now being tested should give positive results with normal serum; if the inhibitory action is not due to antibodies, then the agar gel diffusion antigen-antibody titration technique should yield negative results as indeed was found to be true.

Bands of precipitation were found only between the protease antigen and the specific antiserum. No bands were demonstrated between the normal serum and the protease antigen.

Another factor which could account for inhibition of proteolytic activity by normal serum suggests that the serum proteins may be competing as a substrate for the enzyme; therefore, in higher concentrations of serum the serum protein may have a greater affinity for the enzyme than does casein, or the serum protein may be in greater concentration thus effectively tieing up the enzyme. We have previously shown that the Pseudomonas protease does degrade albumin.[2] However, the activity when albumin is substrate is much less than when casein or corneal protein fractions serve this purpose. The albumin in the serum may therefore be effectively binding the enzyme sites, due to its slow activity.

Another possible factor inhibition of protease activity is the presence of an inhibitor against the enzyme in normal serum. Some investigators have found that inhibitors against enzymes are present in normal serum and play a role in reducing enzyme activity especially at the lower dilutions tested. In any case, the evidence presented in this paper suggests the presence of antiserum antibody which inhibits the proteolytic activity of the enzyme over and above that observed for normal serum. Further studies, therefore, with the specific antiserum may lead to successful therapy in prevention of corneal damage during Pseudomonas ocular infections.

Summary

The Pseudomonas protease has been shown actively to degrade casein, hemoglobin, tendon collagen, and two different corneal protein fractions. One of these corneal fractions is thought to be related to corneal

collagen and it is believed that enzyme has collagenase activity which may be related to the corneal damaging factor as observed when the enzyme is inoculated intracorneally into the eyes of rabbits.

Immunologic studies indicate that antibodies may be produced against the protease. These antibodies inhibit the proteolytic activity of the enzyme. It is hoped that these observations may prove fruitful in prevention of corneal damage during Pseudomonas ocular infections.

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ACKNOWLEDGMENTS

We wish to acknowledge the able technical assistance of Mr. Charles Catanese.

REFERENCES

1. Fisher, E., Jr., and Allen, J. H.: Corneal ulcers produced by cell-free extracts of Pseudomonas aeruginosa. Am. J. Ophthal., **46**:21 (July, Pt. II) 1958.
2. Fisher, E., Jr.: Protease produced by Pseudomonas aeruginosa. Fed. Proc., **17**:510, 1958.
3. Kunitz, M.: Crystalline soybean trypsin inhibitor. J. Gen. Physiol., **30**:291, 1947.
4. Guidry, M. A., Kelly, J. B., and Allen, J. H.: Comparative study of soluble proteins of corneas from three mammalian species. Proc. Soc. Exper. Biol. & Med., **92**:469, 1956.
5. Boyd, W. C.: Fundamentals of Immunology. New York, Interscience Publishers, 1956, ed. 3.
6. Feinberg, J. G.: A new quantitative method for antigen-antibody titration in gels. Nature, **177**:530, 1956.

DISCUSSION

DR. SEYMOUR HALBERT (New York): This demonstration of the production of collagenase by Pseudomonas organisms is of extreme importance, and it must be stressed here that collagenase is a proteolytic enzyme which is capable of hydrolyzing collagen as it exists in the tissues.

Other proteolytic enzymes, trypsin, chymotrypsin, or any of the others that have been studied, cannot do this. Collagenases have been described as products of Clostridia, especially Clostridium perfringens. These organisms cause gangrene; and these collagenases have been studied and in some instances purified to a very high degree.

There is extremely strong circumstantial evidence, in the case of the gas gangrene-producing organisms, that the enzyme, collagenase, does play a major role in the pathogenesis of the disease. If one injects small quantities of the purified clostridial collagenase preparations into the skin, one gets rapid destructive ulcerating lesions, somewhat similar to the ones that have been shown here.

Dr. von Sallmann some years ago, in unpublished work, injected minute amounts of purified clostridial collagenase into the vitreous of rabbit eyes and got extremely destructive changes. If the pseudomonas organisms actually do produce collagenase, therefore, this enzyme could very well play an important role in the infectious process by this agent.

There are, however, a few words of caution that must be given in regard to these observations. Because of the significance of the detection of collagenase, it is extremely important to be absolutely sure that the tendon collagen preparation that was used is really pure native collagen, that is, is resistant to hydrolysis with trypsin chymotrypsin, and so on. I would like to ask the author whether these tests have been done with the tendon collagen preparations they used.

The difficult task of purifying the enzyme is complicated by the possibility that several proteolytic enzymes may exist in these culture materials that have been used. This certainly has been found to be the case in the clostridial studies that have been carried out. One might check for the multiplicity of enzymes by careful assays at many different pH levels, since frequently these different proteolytic enzymes have slightly different pH optima. Also, some proteinases require reducing conditions, and one might be able to detect differences in activity if the hydrolytic properties are tested in the presence of something like cysteine, a reducing substance.

With regard to the immunologic data, one must also be very cautious about drawing conclusions. Here in normal rabbit serum, apparently, there are inhibitors that interfere with at least some of the proteolytic enzymes that are present in these culture preparations, and it would be extremely important always to use pre-immune sera from the same rabbit that was immunized for the production of antibody as a control so that one knows what was present before and what was present after immunization. In addition, the use of gamma globulin fractions would be important, because it is possible that the normal inhibitor may not be present in the gamma globulin fractions, and therefore one could actually study uncomplicatedly the antibody to the preparation.

One must also keep in mind the possibility that antibody to the enzyme may be produced but that it may not interfere with the enzyme activity itself. This kind of thing has been shown with many other anti-enzyme systems.

DR. EARL FISHER, JR. (closing): I would like to thank Dr. Halbert for his kind comments, and add that we have tried enzymes such as trypsin in the cornea and have not obtained the same effects as those observed with the Pseudomonas enzyme.

In our purification procedures we use ammonium sulfate fractionation and recently I have found that calcium phosphate gel columns are very useful in purifying the enzyme.

Inhibitor studies have indicated that there is a metal cofactor required for activity of the enzyme. Such things as versene inhibit the activity of the enzyme. Glutathione inhibits the activity of the enzyme. So, I doubt that reducing conditions are necessary for activity.

We might also mention that very recently we have been able to show protection of the corneal tissue in vivo with the antiserum, and this prevents the ulcerative condition which was noted in the third slide.

TOXOPLASMOSIS: THE NATURE OF VIRULENCE*

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Variation in virulence from strain to strain is a common phenomenon observed in Toxoplasma gondii.[1] Aside from studies which record differences in the pathologic picture produced by certain strains,[2] the main criteria for evaluating virulence have been the percentage mortality of animals given graded inocula of parasites, and the survival time of those that succumb. In general, it appears that, while the species of host is important in relation to such data, the relative order of virulence of different strains seems constant; that is, one strain is more virulent for many hosts and another strain is less virulent. This suggests that for each strain there may be some intrinsic characteristic contributing to its virulence.

Toxoplasma gondii is a relatively large intracellular parasite and can be observed readily within cells by ordinary light microscopy (Fig. 1). It grows readily in a wide variety of tissue cultures and may produce lysis of the cultures.[3] Therefore, it seemed possible to utilize tissue culture techniques to investigate the rate of penetration and attachment of the organisms, the proportion of cells invaded, and the rate of multiplication of virulent and avirulent strains of the parasite. This report presents information on these points in regard to three strains of Toxoplasma gondii.

METHODS AND MATERIALS

The toxoplasmas used were of the RH strain (very virulent for mice),[4] the S_3 strain (moderately virulent for mice),[5] and the 113 CE strain (relatively avirulent for mice).[6] All strains were maintained by injection into the yolk sack of chick embryos and were harvested from ground chick chorioallantoic membranes. For each experiment the Toxoplasmas were obtained simultaneously from live embryos. Suspensions of organisms were counted in a Neubauer-Levy counting chamber and were diluted with tissue culture media until the desired inoculum was obtained. These manipulations were performed within one to two hours to avoid attrition of viable organisms.

Monkey kidney tissue cultures were maintained in medium consisting of Hank's balanced salt solution, sodium bicarbonate, lactalbumin hydrolysate, and two-percent calf serum. Human amnion cell cultures, as previously described, were maintained in Eagle's basic medium with glutamine and two-percent calf serum.[7] Both roller tubes and "flying coverslips" were used. They were maintained at a temperature of 37°C., and the medium was changed as required. To the media of all cultures, penicillin, 100 units per cc., streptomycin, 100 µg. per cc., and

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mycostatin, 50 units per cc., were added. In a given experiment the same batch of tissue culture tubes was used for all strains. After inoculation the time permitted for penetration of the organisms was controlled by decanting the supernatant fluid containing whatever parasites were still free. The tissues were washed two times with uninfected medium and the tubes were then reincubated with fresh medium. After reincubation for a time, sufficient for multiplication of the parasite, but not for cell lysis, the medium was decanted. All cultures were fixed in Bouin's fixative; and the roller tube cells were imbedded in a collodion sheet.[7] The tissues were then stained with hematoxylin and eosin. At the time of fixation, the medium from each tube was centrifuged and the sediment was examined for organisms. If organisms were present, this was taken as an indication that cell lysis had occurred, and the tubes were discarded.

The same investigator examined all specimens, and had no previous knowledge of the strain being studied. Consecutive cells were observed under 800 to 1250 times magnification. The percentage of cells infected and the number of organisms per cell were tabulated.

Results

The lysis times of monkey kidney tissue cultures infected with comparable numbers of the three strains studied are given in Table 1. In tissue culture, as in animals, the RH strain is the most virulent and results in the most rapid lysis of the cultures, the S_5 strain is intermediate, and the 113 CE strain is so avirulent as to cause only erratic cell lysis. In orded to study the factors responsible for the marked differences in virulence shown, the experiments reported in Table 2 were performed.

TABLE 1

Lysis time of monkey kidney tissue cultures

Time of Attachment of Organisms (hr.)	RH Strain (da.)	113 CE Strain	S_5 Strain (da.)
½	5	0 lysis, 3 wk.	—
1	4	0 lysis, 3 wk.	—
3	4	2 tubes, 12 da.	10
		3 tubes, 0 lysis, 3 wk.	—
26	4	2 tubes, 11 da.	—
		3 tubes, 0 lysis, 3 wk.	—

Experiments 1 and 2 indicate that the number of cells parasitized increased roughly with the time of exposure of the cells to the organisms during the first three hours. Following this, the rate of invasion by the parasite decreased, presumably because of loss of viability of parasites remaining extracellularly and a decreasing number of organisms in the supernatant fluid.

Experiments 3, 4, and 5 provide additional data on the percentage of cells invaded within three hours of exposure, and on the rate of reproduction of the parasites within the cells. Most striking in all experiments was the marked difference in the percentage of cells invaded after exposure to equal inocula of different strains. The average percentage of cells infected by the virulent RH strain after three hours of exposure of the roller tube cultures to organisms was 22.92. The average percentage of cells infected by the relatively avirulent 113 CE strain was only 4.49, and the S_5 strain invaded 11.3 percent of cells. The same number of organisms in a rolling tube with coverslips invaded fewer cells than in a stationary monolayer tube.

Not only differences in invasiveness but also differences in the rate of multiplication between the RH and 113 CE strains were very significant to $P = < 0.01$. The average number of RH Toxoplasmas per cell after incubation was 11.83; whereas the average number of 113 CE Toxoplasmas per cell was 3.86. The intermediate S_5 strain showed 9.00 Toxoplasmas per cell (table 3).

These differences are illustrated by comparing Figure 1 and Figure 2. The greater infectivity of the RH strain, and the relatively large number of organisms per cell compared to the 113 CE strain, is apparent.

Assuming that the organism divides at a

TABLE 2

COMPARISON OF INVASIVENESS AND RATE OF REPRODUCTION OF THREE STRAINS OF TOXOPLASMA GONDII IN TISSUE CULTURES

			RH Strain		S_6 Strain			113 CE Strain	
	Penetration Time		% Cells Infected	Toxo/cell	% Cells Infected	Toxo/cell		% Cells Infected	Toxo/cell
Experiment I									
Inoculum: 150,000 parasites. Monkey kidney cells in stationary tubes. Cultures incubated 26 hours after inoculation; 300 cells counted per culture.		(1)	3.3	17.5				2.67	8.2
	½ hr.	(2)	1.67	16.0				1.0	12.3
		(3)	3.00	12.8				2.33	6.1
	Average		2.66	15.4				1.99	8.87
		(1)	4.67	10.4				3.0	4.4
	1 hr.	(2)	6.30	14.5				3.3	4.8
		(3)	6.67	12.9				4.0	7.0
	Average		5.88	12.6				3.43	5.4
		(1)	33.0	15.7				2.33	9.7
	3 hr.	(2)	20.0	12.8				4.0	7.0
		(3)	22.0	14.0				2.0	3.0
	Average		28.3	14.2				2.78	6.57
	26 hr.	(1)	31.0	15.0				6.33	7.36
		(2)	33.0	16.3				4.0	5.0
	Average		32.0	15.61				5.17	6.2
Experiment II									
Inoculum: 150,000 organisms. Monkey kidney cells on flying coverslips in a roller drum. Cultures incubated 24 hours after inoculation. 600 cells counted per culture.	½ hr.	(1)	1.33	6.62			(1)	1.5	1.0
		(2)	1.65	9.78			(2)	0.77	1.5
							(3)	0.77	1.0
	Average		1.49	8.20					
							Average	1.01	1.17
	1 hr.	(1)	1.50	8.4			(1)	2.0	1.5
		(2)	1.84	6.35			(2)	1.3	1.28
							Average		
	Average		1.67	7.37				1.6	1.39
	3 hr.	(1)	4.33	7.54			(1)	2.67	1.45
		(2)	8.0	7.1			(2)	3.0	1.67
	Average		6.17	7.32			Average	2.83	1.56
Experiment III									
Inoculum: 400,000 organisms. Monkey kidney cells on flying coverslips in a roller drum. Cultures incubated 25 hours after inoculation	3 hr.		3.78	11.82				1.	1.3
			2.78	10.4				1.	2.0
			3.78	10.46				0.67	2.0
			2.33	10.14				0.67	3.0
	Average		3.17	10.71				0.84	2.1
Experiment IV									
Inoculum: 300,000 organisms. Human Amnion Cells in stationary tubes. Cultures incubated 24 hours after inoculation.		(1)	20.3	12.3				2.4	1.8
	3 hr.	(2)	23.5	10.5				4.0	2.7
		(3)	22.5	10.9				3.2	1.8
	Average		22.1	11.3				3.2	2.1
Experiment V									
Inoculum: 230,000 organisms. Monkey kidney cells in stationary tubes. Cultures incubated 26 hours after inoculation.	3 hr.		24.5	10.5	11.5	10.7		6.	2.5
			22.	14.0	11.5	7.2		7.5	3.67
			18.5	14.6	11.	9.		9.	3.02
	Average		21.7	13.0	11.3	9.00		7.5	3.1

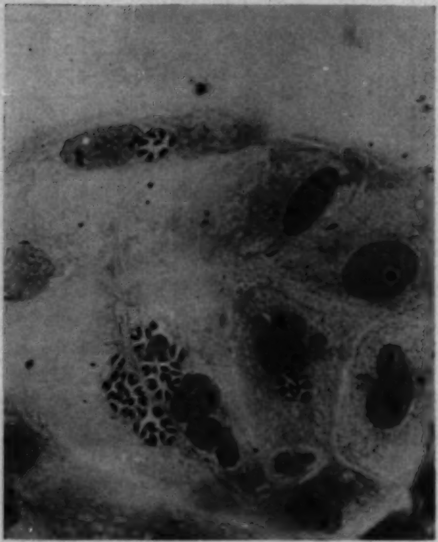

Fig. 1 (Kaufman, et al.). A photomicrograph of a typical monkey kidney tissue culture monolayer infected with 100,000 RH (virulent) Toxoplasmas, incubated for 26 hours after infection, and stained with hematoxylin and eosin. Easily seen are the many cells invaded, the large number of organisms per cell, and the large individual rosettes.

uniform rate, by binary fission, the average generation time for the RH strain was seven hours. That for the 113 CE strain was 15 hours and 40 minutes, and for the S_s strain was seven hours and 25 minutes. The difference in the mean rate of multiplication between the virulent RH and avirulent 113 CE strains of Toxoplasma is striking, though even within a single strain there was some variation in the rate of multiplication.

The fact that the number of organisms per cell did not greatly increase with increasing exposure and greater invasion of the tissues suggests that invasion of a cell by more than one organism was not an important factor in explaining these differences in multiplication. In addition, when single rosettes of organisms were observed, those of the RH strain were comprised of many more parasites than those of the 113 CE strain.

TABLE 3

RATE OF MULTIPLICATION

RH/113 CE	Ratio of rate of multiplication 2.25 X
RH/113 CE	Ratio of number of organisms per cell after 25 hours 3.65 X

The greatest number of organisms seen after 25 hours in an RH infected cell was 41 and in an 113 CE infected cell was 21. The minimal division time for RH was four hours and 20 minutes, and for 113 CE was five hours and 40 minutes.

When lysis occurred and sections were stained, the virulent and avirulent strains had similar numbers of organisms within the cells. Although quantitation was not possible, this suggests that premature destruction of the cell and early liberation of organisms was not important for the differences in virulence observed.

DISCUSSION

Virulence is a complex phenomenon un-

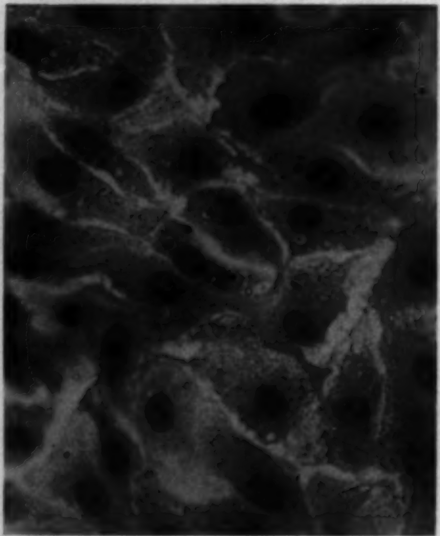

Fig. 2 (Kaufman, et al.). A photomicrograph of a typical monkey kidney tissue culture monolayer infected with 100,000 113 CE (avirulent) Toxoplasmas, incubated 26 hours after infection, and stained with hematoxylin and eosin. Only a small clump of organisms is visible.

derstandable only in terms of host-parasite interaction. Presumably in these tissue culture studies there is no antibody produced. Only the "inherent resistance" of the cell cultures and properties intrinsic to the various strains of organisms contribute to the results obtained.

Although some strains of organisms in vivo are more lethal than others, and although there is some evidence that virulent strains may be slightly larger in size than avirulent strains,[8] no quantitative antigenic, enzymatic, or other difference explaining this strain distinction has been reported. Moreover, several authors have described increases in virulence of given strains with continuous animal passage.[6, 9-11] The nature of this adaptation or change is obscure.

The results presented indicate that the virulence of Toxoplasma in tissue culture is parallel to the reported virulence in animals. One hundred RH Toxoplasmas kill mice in eight days, 113 CE kills mice in 23.8 days, and S_5 is lethal to mice in 10.9 days.

Correlated with virulence is both the invasiveness and the rate of multiplication. For example, the RH strain invades 5.1 times the number of cells that are invaded by the 113 CE strain. It seems that a smaller percentage of 113 CE organisms are able to invade and thus a larger inoculum may be necessary to cause clinical infection. The time required for attachment of the parasite would favor infection of structures with relative end arteries, such as the retina, as opposed to structures with a widely anastomosing vascular bed and rapid blood flow such as the choroid.

The difference in the rate of intracellular multiplication by the different strains is striking. The determinants of these differences in the rate of multiplication may be crucial in influencing the efficacy of our present anti-Toxoplasma chemotherapy. Daraprim is a metabolic antagonist active as a competitive inhibitor in the folic acid pathway of metabolism; sulfa drugs competitively antagonize paramino benzoic acid metabolism. In general, as exemplified in the chemotherapy of cancer, the antifolic drugs and other metabolic antagonists are more effective against more rapidly dividing cells. In Toxoplasmas also, the differences in rate of multiplication may be associated with differences in drug sensitivity, and the slowly multiplying strains may be relatively resistant. Preliminary investigation supports this hypothesis. The relative degree of resistance of more slowly growing organisms and the relation of this resistance to in vivo drug levels are now under study.

These findings with directly observable intracellular parasites may point to similar characteristics in viruses, and clarify phenomena such as variable responses to antimetabolite drug therapy and the nature of chronic infection.[12]

Summary

Studies of different strains of Toxoplasma gondii in tissue culture indicate that virulence is correlated both with an increased proportion of cells invaded and a more rapid rate of multiplication of the organisms. The application of these observations to problems of infectivity and differences in the resistance of various strains to drug therapy is discussed.

National Institute of Neurological Diseases and Blindness (14).

References

1. Jacobs, L.: Propagation, morphology, and biology of Toxoplasma. Ann. New York Acad. Sci., **64**: 154-179, 1956.

2. Frenkel, J. K.: Host, strain, and treatment variation as factors in the pathogenesis of toxoplasmosis. Am. J. Trop. Med. & Hyg., **2**:390-415, 1953.

3. Cook, M. K., and Jacobs, L.: The cultivation of Toxoplasma gondii in tissue cultures of various derivations. J. Parasitol., **44**:172-182, 1958.

4. Sabin, A. B.: Toxoplasmic encephalitis in children. J.A.M.A., **116**:801-807, 1941.

5. Jacobs, L., and Melton, M.: A procedure for testing meat samples for Toxoplasma, with preliminary results of a survey of pork and beef samples. J. Parasitol., **43** (Suppl.):38-39, 1957.

6. ———: Modifications in virulence of a strain of Toxoplasma gondii by passage in various hosts. Am. J. Trop. Med. & Hyg., **3**:447-457, 1954.

7. Kaufman, H. E., and Kaufman, E.: Human amnion-cell tissue culture. Arch. Ophth., **59**:342-348, 1958.

8. Harboe, A., and Ericksen, S.: A comparative study of the length of the parasites of four strains of Toxoplasma gondii. Acta. Path. et Microbiol. Scand., **37**:31-42, 1955.

9. Sabin, A. B., and Olitsky, P. K.: Toxoplasma and obligate intracellular parasitism. Science, **85**:336, 1941.

10. De Rodaniche, E.: Susceptibility of the marmoset Marikina Geffreyi and the night monkey Aotus zonalis to experimental infection with Toxoplasma. Am. J. Trop. Med. & Hyg., **3**:1026-1032, 1954.

11. ———: Spontaneous toxoplasmosis in the whiteface monkey Cebus capuncinus in Panama. Am. J. Trop. Med. & Hyg., **3**:1023-1025, 1954.

12. Matthews, R. E., and Smith, J. D.: The Chemotherapy of Viruses: Advances in Virus Research, Vol. III. New York, Acad. Press, 1955, pp. 51-142.

Discussion

Dr. Michael J. Hogan (San Francisco): This is an important study, designed to demonstrate the growth and activity of Toxoplasma. Dr. Kaufman and his co-workers, faced with the necessity of presenting a fair amount of information, would not be able in this session to emphasize other important findings which are presented in their paper.

It was observed very early that strains of Toxoplasma varied somewhat in virulence, and a great deal of work was done to elucidate various aspects connected with this phenomenon. At the present time we are still unaware of the mechanism which determines virulence.

The authors have shown by this tissue culture method that the virulent RH strain is more capable of rapid multiplication, invades a higher percentage of cells, and produces many more intracellular organisms than strains of lesser virulence. We must be content with the fact that such differences in strains exist. Presumably these differences account for the varying clinical picture presented by patients with toxoplasmosis, including the eye forms.

This past year we isolated a strain of Toxoplasma from the chorioretinal tissues of a 20-year-old boy who had congenital toxoplasmosis. The strain that was isolated was of low virulence, and required some time to become manifest and be recovered from mice. One might ask if these organisms might not have become attenuated over a long period of years after residing in the ocular tissues. The other assumption is that these organisms originally possessed low virulence and produced such a mild disease in the fetus that its presence was not suspected.

The real value of this paper is its proof of the action of various strains on cells. By such studies the authors may be able to show what the principal factors are in determining the type of toxoplasmic infection that occurs in humans. The observations made in these tissue culture studies correlate well with the findings which are seen in animals on experimental inoculation of these same strains.

The authors' observations of the localization of organisms in tissues which have end arteries are quite interesting to me. One would expect in such eyes that possibly the disease should localize more toward the terminal branches of these end arteries. In other words, the higher percentage of ocular lesions should be toward the terminal branches, whereas most of them are in the posterior fundus where the vessels are larger. However, the finer branches of these large vessels may determine the position of the lesions. Some organisms do get into the terminal circulation and produce lesions, although such an observation is rare in human eyes, and in experimental infections.

The suggestion that pyrimethanine and sulfadiazine act best against those organisms which reproduce rapidly, and presumably have a much higher metabolic activity, is of great interest, and this may have a tremendous bearing on the results of treatments with sulfadiazine and pyrimethamine in ocular disease. Possibly in some cases we are not dealing with organisms which are multiplying rapidly or invading cells rapidly. Perhaps the organisms we are dealing with have a low metabolic activity and do not take up these chemical agents.

There is a considerable viriation in the response of toxoplasmic infections to chemotherapeutic agents, and we have not as yet been able to explain why the variation should occur. For example, in a study of patients with undoubted ocular toxoplasmosis, one case had no response over a period of one month's treatment with pyrimethamine and sulfadiazine.

This is a nice study, and we agree with the methods pursued. In case some might wonder, we had a few questions which the authors were able to explain. Fisher and his co-workers in 1954 showed that the relationship between the number of Toxoplasma and the number of cells in the tissue culture was rather important. For example, if there were three Toxoplasma for every five cells in the tissue culture, there was a 19-hour release of organisms from the cells. If there was a proportion

of one Toxoplasma per 10 tissue culture cells, there was a 43-hour release, or a much delayed release of organisms from the cells. However, Dr. Kaufman pointed out to me that he is dealing with monkey kidney cultures, which are very different from the ones used by Fisher and his co-workers.

It is not possible, on a quantitative basis, to assay the relationship between the number of organisms in the supernatant fluid and those in the cells, so this method of pursuit of the problem would not be of any great value.

We have enjoyed very much having an opportunity to review this paper, and are looking forward to further studies by these three investigators.

Dr. Seymour Halbert (New York): I would like to ask two questions. One is whether antisera to the various strains have been tested, and whether they are equally protective. One might suspect that the difference in virulence could perhaps be associated with different antigens of one sort or another, and that antisera against the various strains might reflect these differences.

Secondly, I would like to recall that recently Bernheimer, in New York, has shown with Group A hemolytic streptococci, when ingested by leukocytes, that certain strains of the streptococci will cause lysis of the neutrophils after a short length of time. He has shown very nicely an excellent correlation between the capacity of these strains of streptococci to produce an enzyme which destroys diphosphopyridinenucleotide, an important coenzyme, and the leukotoxicity. I would like to raise the possibility that perhaps the strains of Toxoplasma which cause lysis more rapidly than the avirulent strains may be associated with the production by these Toxoplasma of a similar enzyme.

Dr. Herbert E. Kaufman (closing): I would like to thank Dr. Hogan and Dr. Halbert for their kind, constructive, and very interesting comments. I appreciate the benefit of Dr. Hogan's great experience in this field.

Moderately extensive tests of antibodies against the different strains of Toxoplasma have been made. It has been found that infection with a virulent strain usually gives a higher titer of antibody, but there have been no qualitative differences in antibody type or function demonstrated as yet. As far as is known, the antibodies produced to all strains are identical, but the precise role of these circulating antibodies in producing immunity is not certain.

As to the second question, when one uses phagocytic cells and bacteria with proteolytic enzymes the situation becomes very complex. There is evidence in tissue cultures that Toxoplasmas are toxic to the cells mainly in terms of their mass effect. Once the cell is infected, it continues living, moving, and dividing. As one looks at the culture, the cell becomes full of organisms, and seems to burst only when completely filled. As far as I know, there is no evidence to suggest a primary proteolytic process in causing lysis of the cell, but such a process, in regard to penetration of the cell by the organism and lysis of the cell, has not been ruled out.

STUDIES ON CHRONIC TOXOPLASMOSIS*

The relation of infective dose to residual infection and to the possibility of congenital transmission

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The possibility of the occurrence of congenital transmission of toxoplasmosis in children of women with chronic or latent infections has been the subject of considerable conjecture and some experimentation. It remains a debatable subject despite the fact that women who have borne toxoplasmic children, and who have been followed subsequently, have been found to have as good a chance as other women in the general population of bearing normal children in subsequent pregnancies.[1,2] Certain clinical observations, such as those of Campbell and Clifton,[3] Campbell,[4] and Seitelberger and Spiel,[5] have led these workers to consider transmission from the chronic state as a possible explanation for what they think are repeated cases of congenital toxoplasmosis in the same family. Ophthalmologic observations, such as those of Johnson,[6] point to the reactiviation of toxoplasmic chorioretinal lesions during pregnancy. Other reports of cases of chorioretinitis in children indicate that sometimes this symptom is the only finding in congenital toxoplasmosis.[2,7] The idea naturally suggests itself that maternal

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immunity must play a large part in the development of the fetal infection, and that localized, as contrasted with disseminated infection in the infant, may be the result of the rapid rise in antibody levels which would occur by recall in a mother with a reactivation of latent parasites.

Weinman[8] has expressed this view concerning human toxoplasmosis, and other workers have reported observational and experimental data on the occurrence of toxoplasmosis in offspring of chronically infected animals.[9-12] Some details concerning these reports will be discussed below. There is one point, however, in the work of Hellbrügge[11] which has particularly attracted our attention.

Hellbrügge found that congenital transmission occurred regularly in rats with chronic infections produced originally by intravenous injection of 3×10^6 parasites, but not in similar animals that received only 3×10^3 Toxoplasmas. He assumed that in the massively infected animals there is an exacerbation as a result of pregnancy and that parasitemia developed because of it; also, that such an exacerbation did not occur in rats given small numbers of organisms.

This is in itself an interesting observation. In essence, it suggests that the degree of residual infection varies depending on the size of the inoculum. This relation holds with metazoan parasites which merely mature in the definitive host, but it is usually not considered in studies of parasites which can proliferate until the host's defenses are mobilized against them.

The present investigation was undertaken with the idea of gathering some definitive information on the frequency with which Toxoplasma can be transmitted congenitally from rats with chronic infections and under what circumstances. In view of Hellbrügge's work, we were especially interested in the relation of the size of the inoculum to: (1) the degree of residual infection, (2) antibody-formation, and (3) distribution of parasites in various organs, as well as to (4) the occurrence of congenital transmission.

Materials and Methods

The RH strain of Toxoplasma gondii was used in all of the experiments to be reported. Mice infected intraperitoneally three days previously served as the source of peritoneal exudate from which inocula were prepared. The parasites were counted in a 1:100 dilution of the exudate, with the use of a Neubauer-Levy hemacytometer. Appropriate dilutions were then made to prepare inocula containing the calculated numbers of parasites desired. The dilution fluid was 10-percent inactivated normal rabbit serum in saline, and all inoculations were completed within one hour.

Females of the Sprague-Dawley strain of rat were injected either intracardially or intraperitoneally with the inocula. Male rats of the same strain served for breeding purposes only and were not infected. Rats of this strain, weighing 120 or more grams, generally survived intraperitoneal inoculation with 1×10^6 Toxoplasmas, but one third to one half of the animals succumbed to 1×10^8 parasites.

Determination of chronic infection

Six to eight weeks following infection, rats were killed and their organs tested for the presence of Toxoplasma. The entire brain, heart, lungs, and spleen were weighed individually and 1:10 (wgt./volume) suspensions of these organs were prepared by grinding the organs in mortars and adding the appropriate amount of saline. Two grams of liver and 10 grams of muscle were similarly triturated in a volume of saline 10 times their weight. One ml. of a 1:10 suspension of each organ was inoculated intraperitoneally into each of five mice.

The mice used were of the NIH "general purpose" and NIH strains. They weighed between 18 and 20 gm. In studies of hundreds of mice by Perrin[22] and by us, NIH mice have not been demonstrated to have spontaneous Toxoplasma infections.

For the experiments on congenital transmission, 120- to 150-g. rats were inoculated intraperitoneally with various numbers of

organisms. Six to eight weeks after infection, they were placed with males of the same strain and allowed to mate. No attempt was made to determine the day of conception and the females were removed from the males only when enlargement of the abdomen could be seen. They were then placed in separate cages. Variations in the method of handling the offspring will be described below in the appropriate section.

The mice injected with suspect rat tissues were examined daily for signs of illness. In some instances death of mice inoculated with concentrated rat brain suspensions occurred within 24 hours, possibly due to thromboplastin.[13] When mice died three or more days after inoculation, smears of peritoneal fluid were studied microscopically. If no parasites were found, Giemsa-stained impression smears were made of the brain, liver, and spleen; and portions of these organs were also triturated together and inoculated into mice.

Deaths of mice usually occurred between seven and 21 days after inoculation with tissues from chronically infected rats. In the first experiment on residual infection, the dye test for toxoplasmosis was performed on a random sample of the mice to determine if any infections took place that did not cause death of the animals. This was known to be very unlikely since it has been found that the RH strain is highly virulent for mice even when inocula calculated to contain single organisms are prepared. From those mice remaining alive in the other experiments, three, or less if fewer remained, were bled and their serums pooled for dye-testing. If the dye test was positive, organs of the remaining mice were subinoculated in order to demonstrate the parasites.

Dye tests were also performed on the serum of mother rats. The dye test technique used has been described in detail elsewhere.[14] The method of bleeding the animals was that originally described by Pettit[15] and more recently by Halpern and Pacaud.[16] Pasteur pipettes were inserted past the inner canthus of the eye into the orbital sinus. Sufficient amounts of blood were forced into the pipette by the positive pressure in the sinus. Rats could be bled on alternate days for a week or more with no mortality; at each bleeding at least one ml. of blood was taken from both rats and mice.

Certain additional details of procedure will be given below in the appropriate sections.

Results

Residual infection

Rats were infected with inocula calculated to contain 1×10^3, 1×10^6, or 1×10^8 Toxoplasma. Six to eight weeks later, these rats were killed, and their brains were tested for infectivity by inoculation of a 1:10 suspension into mice. The results are summarized in Table 1. It appears, from the number of mice which succumbed to toxoplasmosis, that there is definitely more residual infection in the animals that receive larger inocula of parasites. A larger study of rats by the same methods but in addition with titrations of brain suspensions is presently under way, and the results, although incomplete, seem to confirm these findings.

In addition to the brain, the following organs of the rats in the above series were tested for the presence of parasites: liver, spleen, heart, lung, and skeletal muscle. Ex-

TABLE 1

Degree of residual infection in relation to size of inoculum

No. of Rats	Size of Inoculum	No. Showing Residual Infection	Composite Results of Inoculation of 1:10 Dilution of Brain into Mice		
			No. of Mice	No. Dead	Survival Days (and range)
3	1×10^3	0	15	0	——
5	1×10^6	3	15	7	11.1 (11–12)
3	1×10^8	3	15	12	7.8 (7–12)

TABLE 2

SEROLOGIC RESPONSE OF RATS FOLLOWING INOCULATION WITH VARIED NUMBERS OF TOXOPLASMA GONDII

Series No.	Rat No.	Size of Inoculum	Reciprocal of Titer on Designated Day of Infection*										
			2	3	4	5	6	7	8	9	10	11	12
I	1 & 3	1×10^3		0			16			2,000			4,000
	2 & 4	1×10^3		0			0			2,000			1,000
	5 & 7	1×10^6		4			1,000			4,000			4,000
	6 & 8	1×10^6		16			1,000			4,000			8,000
	9 & 11	1×10^8		64			4,000			4,000			16,000
	10 & 12	1×10^8		64			4,000			4,000			16,000
II	1, 3, 5	1×10^3	0	0	16	0	16	256	64	1,000	4,000	256	
	2, 4, 6	1×10^3	0	0	4	3	8	64	128	1,000	2,000	2,000	
	7, 9, 11	1×10^6	4	64	256	4,000	4,000	16,000	16,000	16,000	16,000	4,000	
	8, 10, 12	1×10^6	4	16	256	256	256	8,000	8,000	4,000	4,000	4,000	
	13, 15, 17	1×10^8	4	16	2,000	4,000	16,000	16,000	16,000	16,000	16,000	16,000	
	14, 16, 18	1×10^8	4	16									

* Day of infection = day 0.

cept for one positive finding in muscle, mice inoculated with suspensions of these organs were all negative; no deaths occurred among them, no parasites were demonstrable in impression smears of their organs, and dye tests performed on a random sampling of some of the mice in each series were negative.

SEROLOGIC RESPONSE IN RELATION TO SIZE OF INOCULUM

Two series of rats were studied as to the time of appearance of antibodies relative to the size of the inoculum. The results are presented in Table 2 and Figure 1.

In series I, rats 1 and 2, and 3 and 4, were bled on days 3 and 9, and days 6 and 12, respectively. The same is true of the other pairs listed for this series. In series II, three pairs of rats were injected with each dose of the parasites, and each pair was bled every third day. For example, the titers obtained on days 2, 5, 8, and 11 are those of rats 1 and 2; titers obtained on days 3, 6, 9, and 12 are from rats 3 and 4; and rats 5 and 6 supplied the data for days 4, 7, and 10.

There was at least a two-day delay in the appearance of antibodies in rats injected with 1,000 Toxoplasmas, in comparison with rats injected with 1×10^6 or 1×10^8 parasites. It is also to be noted that the initial appearance of antibodies was followed by a rapid climb to high titers in the rats given the larger inocula, while the titers remained at a lower level for several days in the animals given the small inoculum.

It thus appears that antibody-production could be expected to inhibit the proliferation of parasites sooner in the heavily infected rats than in the light infections. Whether or not the time lag would be sufficient to allow

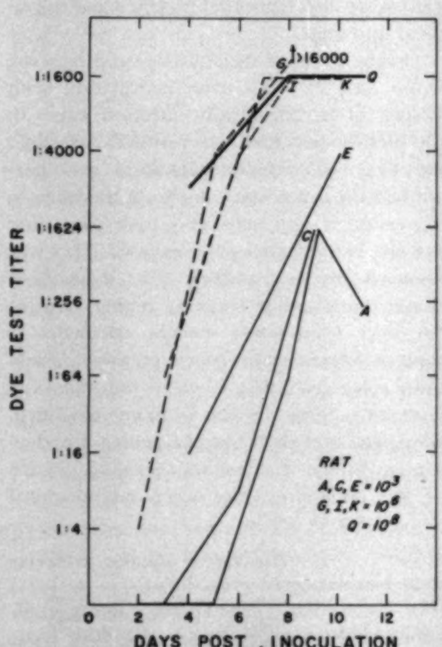

Fig. 1 (Remington, et al.). Serologic response of rats following inoculation with varied numbers of Toxoplasma gondii.

for the development of as large a population in the latter as in the former is a question that must be subjected to mathematical analysis. One complicating factor in resolving this problem is the likelihood that parasites situated intracellularly are not subject to the action of antibody.

Transmission from chronically infected mothers

In the first series of rats studied to test for such an occurrence, 15 female rats were infected intraperitoneally with a 1:10 dilution of peritoneal exudate from mice. This concentration of exudate generally contains between 1×10^7 and 2×10^7 parasites per ml. Each rat received one ml. of the inoculum. Six to eight weeks after infection the rats were mated with uninfected males. The offspring were allowed to suckle and were kept until three to five weeks of age. When they were killed, approximately 2.0 gm. of liver, and the entire spleen were triturated together and one ml. of the suspension was inoculated into five mice. The brain was ground up separately and also inoculated in one-ml. amounts into five mice.

All mice that died following injection with rat tissues were examined for parasites in fresh peritoneal fluid and in Giemsa-stained smears of their organs. In addition their tissues were immediately subinoculated into fresh mice. Dye tests were also performed on pools of serum from three of each group of surviving mice. Dye tests also were performed on the mother rats.

A total of 72 offspring was produced in 15 litters. Three offspring in one litter of nine were found infected with Toxoplasma; the parasites were demonstrated in mice injected with liver and spleen suspension as well as in mice injected with brain material. All the mother rats had positive dye tests, at titers of over 1:1,024. Six of the mothers were also tested for the presence of parasites by injection of 1:10 suspensions of their brains into mice; all six were positive.

This experiment was designed to provide every opportunity for transmission of the infection to the offspring, and therefore the young were allowed to suckle. It is not possible definitely to attribute the presence of Toxoplasma in the single positive litter to congenital transmission. The possibility exists that the milk may have been the source of the infection.

In the next series of rats, some of the young were allowed to suckle and some were killed immediately and their tissues tested as described above. This series comprised seven mother rats which had received 1×10^7 Toxoplasmas intraperitoneally six to eight weeks before being bred. These rats yielded 68 offspring. Thirty-eight of these were killed at birth and 30 were allowed to suckle for one to two weeks. None of the young was found positive for Toxoplasma.

The mother rat that had produced positive offspring in her first litter was allowed to breed again. Three offspring were allowed to suckle and the remaining five of the second litter were killed at birth. None of the young was found to have toxoplasmosis.

In an additional series the uteri and ovaries of chronically infected rats were tested for the presence of Toxoplasma. The uterus was triturated with sand and four ml. of saline was added; one ml. was injected intraperitoneally into each of four mice. The ovaries were similarly treated with three ml. of saline and one ml. was injected into each of three mice.

The 24 rats used were as follows: The seven mothers from series II, just described; 11 rats that had received 1×10^8 parasites but that had not been bred; three rats that had received 1×10^6 parasites and had been bred, and three unbred rats that had received 1×10^3 Toxoplasmas.

Two residual infections in the reproductive organs were found by these techniques. The uterus of one mother and the ovaries and uterus of another of the mothers of series II were positive. It is noteworthy that these rats had delivered litters of 10 and 11 uninfected young, respectively.

Discussion

The results of our tests are in agreement with those of Thiermann,[17] who found only occasional transmission of toxoplasmosis from chronically infected mother rats to their offspring. They agree less with the reports of our German colleagues,[9,10] who found such transmission to occur more frequently.

In contrast to the observations of Cowen and Wolf on mice,[18] Hellbrügge, et al.,[11,12] assume that the presence of organisms in the placenta of rats is due to their presence in the circulating blood rather than to local proliferation. On the other hand, Cowen and Wolf claim that local proliferation must take place on the maternal side of the placenta in mice before parasites can pass into the fetus.

If local proliferation were required, it seems hardly likely that congenital transmission could take place from chronically infected mothers, because proliferation has not been demonstrated to occur in the presence of an immune response. While it cannot be denied that occasionally masses of Toxoplasmas may rupture out of the cell walls containing them, there is no evidence of continued proliferation of the parasites in chronic infections except in certain special sites such as the brain and eye, where physiologic barriers delay passage of antibody. The hypothesis of Weinman and of Hellbrügge that exacerbation of latent toxoplasmosis occurs during pregnancy is not supported by evidence of proliferation in the extraneural viscera, even though Hellbrügge did report low parasitemias in the mothers.

Our finding that the uterus of rats may contain latent parasites indicates an alternative explanation for the occurrence of congenital toxoplasmosis in offspring of mothers with chronic infections. The dormant parasites within a pseudocyst may be transported to the fetal tissues when the placenta erodes that portion of the uterus containing them. No parasitemia in the mother or local proliferation of parasites would be necessary. The extent of proliferation of parasites in the fetus would depend on the titer of passively transferred antibody and of accessory factors.

This hypothesis would explain the fact that litters are not uniformly infected. Our single positive finding was of three young in a litter of nine. Wildführ had similar results, with single offspring positive. It is noteworthy that the two rats whose uteri were positive had given birth to normal litters; the attachments of the placentae in these cases were probably such that the dormant parasites in the uteri were not disturbed.

The frequency with which Toxoplasma can localize and persist within uterine tissue probably varies with the strain of the parisite and of the host as well as with the size of the infective dose of organism. Work on other strains of rats and Toxoplasma is now in progress. However, the extrapolation of findings on rats to what can be expected in human infections is of course always difficult. At present, all that can be said is that the available data indicate that *occasionally* toxoplasmosis may be transmitted by a chronically infected rat to her offspring. The answer to the question of the frequency of such an occurrence in human beings may be more easily found by study of populations in which there is a high prevalence of Toxoplasma antibodies even before child-bearing age, such as in Tahiti. The occurrence of residual infection in the uterus may be ascertained from study of post-mortem material from serologically positive women, using the survey technique of Jacobs and Melton.[19]

Sabin, et al.,[20] have stated that the main practical advantage of the diagnosis of congenital toxoplasmosis is that it allows a good prognosis for subsequent children, by distinguishing this infectious disease from a variety of other conditions responsible for congenital defects of the central nervous system. This is based on some follow-up studies by Sabin[21] as well as those already cited above.[1,2] Despite the findings we have reported here, the bulk of recorded clinical evi-

dence still indicates the unlikelihood that a chronically infected woman will produce toxoplasmic children. Our data indicate certain conditions under which a mother might have successive pregnancies complicated by toxoplasmosis. We have no data on the frequency of such conditions, and it would be harmful to negate the thesis of Sabin, et al., without more definitive information. From a statistical standpoint, the obstetrician and pediatrician can still offer to the parents of an afflicted child, the encouragement that they have a good chance of having normal children later.

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REFERENCES

1. Feldman, H. A., and Miller, L. T.: Congenital human toxoplasmosis. Ann. N. Y. Acad. Sci., **64**:180-184, 1956.

2. Eichenwald, H., and Levine, S. Z.: Toxoplasmosis. Postgrad. Med., **15**:282-6, 1954.

3. Campbell, A. M. G., and Clifton, F.: Adult toxoplasmosis in one family. Brain, **73**:281-290, 1950.

4. Campbell, A. M. G.: Toxoplasmosis. Proc. Roy. Soc. Med., **46**:891-898, 1953.

5. Seitelberger, F., and Spiel, W.: Eine besondere Form von Toxoplasmose-Enzephalitis bei zwei Brudern. Wien Ztschr. f. Nervenh., u. d. Grenzgeb., **7**:298-305, 1953.

6. Johnson, L. V.: Use of neutralizing antibody test in the diagnosis of human toxoplasmic choroiditis. Arch. Ophth., **36**:677-684, 1946.

7. Hedenstrom, G.: On the variability of the clinical course of congenital toxoplasmosis with special reference to the occurrence of mild cases. Acta Paediat., **48**:579-584, 1957.

8. Weinman, D.: Toxoplasma and toxoplasmosis. Ann. Rev. Microbiol., **6**:281-298, 1952.

9. Wildführ, G.: Tierexperimentelle Untersuchungen zur Frage der diaplazentaren Uebertragung der Toxoplasmen beim vor der Graviditat infizierten Muttertier. Ztschr. f. Immunitätsforsch., u. exper. Therap., **111**:110-120, 1954.

10. Hellbrügge, T.: Die fetale Infektion in Verlauf der akuten und chronischen Phase bei der latenten Rattentoxoplasmose. Arch. f. Gynakol., **186**:384-388, 392-395, 1955.

11. Hellbrügge, T. F., Dahme, E., and Hellbrugge, F. K.: Tierexperimentelle Beobachtungen zur diaplazentaren Infektion der Toxoplasmen. Ztschr. f. Tropenmed., u. parisitol., **4**:312-322, 1953.

12. Hellbrügge, T., and Dahme, E.: Experimentelle Toxoplasmose. Bemerkungen zur Arbeit von Schultz und Bauer über Placentar-Befunde bei Ratten. Klin. Wschr., **31**:789-791, 1953.

13. Thomas, L.: Studies on the intravascular thromboplastic effect of tissue suspensions in mice: I. The reaction of mice to intravenous injection of a sedimentable tissue component. Bull. Johns Hopkins Hosp., **81**:1-25, 1947.

14. Frenkel, J. K., and Jacobs, L.: Ocular toxoplasmosis: Pathogenesis, diagnosis, and treatment. Arch. Ophth., **59**:260-279, 1958.

15. Pettit, A.: Procede simple pour prelever du sang chez les petits rongeurs. Compt. rend. Soc. Biol., **74**:11-12, 1913.

16. Halpern, B. N., and Pacaud, A.: Technique de prelevement d'echantillons de sang chez les petits animaux de laboratoire par ponction du plexus ophthalmique. Compt. rend. Soc. Biol., **145**:1465-1466, 1951.

17. Thiermann, E. Transmission congenita del Toxoplasma gondii en ratas con infeccion leve. Biologica (Trabajos d. Inst. d. Biol. "Juan Noe," Chile) **23**:59-67, 1957.

18. Cowen, D., and Wolf, A.: Experimental congenital toxoplasmosis. II. Transmission of toxoplasmosis to the placenta and fetus following vaginal infection in the pregnant mouse. J. Exp. Med., **92**:403-416, 1950.

19. Jacobs, L., and Melton, M. L.: A procedure for testing meat samples for Toxoplasma, with preliminary results of a survey of pork and beef samples. J. Parasitol., **43**(Suppl.):38-39, 1957.

20. Sabin, A. B., Eichenwald, H., Feldman, H. A., and Jacobs, L.: Present status of clinical manifestations of toxoplasmosis in man. Indications and provisions for routine serologic diagnosis. J.A.M.A., **150**:1063-1069, 1952.

21. Sabin, A. B.: Complement fixation test in toxoplasmosis and persistence of the antibody in human beings. Pediatrics, **4**:443-453, 1949.

22. Perrin, T. L.: Toxoplasma and Encephalitazoon in spontaneous and in experimental infections in animals. Arch. Path., **36**:568-578, 1943.

Discussion

Dr. Michael J. Hogan (San Francisco): These two papers by the investigators at the National Institutes of Health at Bethesda merit a great deal of attention, because in the first paper they attack the etiologic agent, Toxoplasma, and in this paper they investigate the methods by which congenital toxoplasmosis may be transmitted from the mother to the fetus.

I think most of us are familiar with the early work done on transmission of toxoplasmosis by Wolff and his co-workers. They theorized that possibly toxoplasmosis was transmitted by the mother to the child by extension of organisms through the vagina, thence to the uterus and then to the infant. However, in experimental investigations designed to demonstrate this method of transmission, they were unable to do so.

The next theory was that the disease was transmitted during pregnancy by the mother, who developed an unrecognized subclinical type of acute toxoplasmosis. The mother, it was theorized, developed the disease as a result of infection from an unknown source and presumably developed parasitemia and was able to transmit the disease via the placental circulation to the fetus.

The work of Hellbrügge, cited by the authors, indicated that with very large inocula the disease could be transmitted from the mother to the fetus. The authors however are not able completely to verify the work of Hellbrügge and his co-workers.

This work does demonstrate that in two out of 28 rats there was involvement of the uterus. However, in the litters born of these two rats there were no toxoplasmic infections in the fetuses.

So, we are concerned today with two problems as yet unsolved: First, the method of transmittance of toxoplasmosis from mothers to their offspring, and, second, the reason why pregnant females in humans fail to transmit the disease to a second child after having given birth to a toxoplasmic infant. The authors suggest, however (and this seems most likely to me also), the infection does reside somewhere in the mother, in a chronic latent state. Almost all mothers of toxoplasmic infants fail to give a history or have evidence of an illness which would suggest an acute infection during the course of pregnancy.

This has always been a puzzling thing. The present work suggests that it is possible for a chronic disease in the mother to be lighted up as a result of pregnancy, and that the chronic disease can be transmitted to the fetus from the uterus. An interesting observation is that there are reported examples of congenital ocular toxoplasmosis in the mother in which the disease was flared by a pregnancy. In no instance, as far as I know, has a child ever been infected from such a mother.

There have been no reported cases of acute toxoplasmosis in pregnant women, so we have no evidence as far as transmission to the fetus during the acute stage of the disease in humans is concerned.

I would like to suggest to the authors that they try another experimental animal than the rat, because rats are quite resistant to toxoplasmic infections, and it might be that a more susceptible animal would be a better experimental one for determination of the results of this type of experiment.

As yet there is no definite reason why pregnant female humans fail to transmit the disease to a second child after having given birth to a toxoplasmic infant. I hope the authors will be able to solve this problem as a result of further studies.

It has been a real pleasure to review this paper.

Dr. Jack S. Remington (closing): Thank you, Dr. Hogan, for your suggestions and comments.

As I stated in the paper, we have as yet no proof that chronically infected human mothers can transmit toxoplasmosis to their offspring in utero. In Europe, various studies at obstetric clinics have revealed no such case. However, the problem could certainly be settled more easily by observation of a population, such as Tahiti, where there is considerably more infection (on the basis of serologic surveys) before child-bearing age. The occurrence of a mild form of congenital toxoplasmosis in such a population could be looked for, especially in regard to such a manifestation as chorioretinitis, which has been reported as the sole symptom in a few congenital cases (Eichenwald). It would also be profitable to study mothers and children serologically.

I also mentioned that a survey of the occurrence of residual Toxoplasma infection in the human uterus would lend additional credence to the possibility of the occurrence of congenital transmission during chronicity. Because we found chronic infection in the rat uterus, we recently initiated a survey of uterine infection in women dead of all causes, who showed positive dye tests. We have done five uteri thus far, using the muscle digestion technique that Jacobs and Melton adopted for surveys of meat samples. One uterus has already been found positive. This was from a 57-year-old woman who died of heart disease. Her dye test titer was 1:256. To our knowledge, this is the first report of the isolation of this parasite from the uterus of an adult human.

IN VITRO OBSERVATIONS ON THE BEHAVIOR OF CONJUNCTIVAL AND CORNEAL CELLS IN RELATION TO ELECTROLYTES*

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INTRODUCTION

While many experimentalists have reported on the non-physiologic practice of washing tissues with so-called "normal" saline, the low cost, facility of preparation, and sterilization of 0.9-percent NaCl have kept this solution an essential in the armamentarium of the ophthalmologist. The argument that there is little gross evidence of direct injury from saline washes should not exclude efforts to assess whether subtle changes are brought about and what part this solution may play in the production of complications following surgical intervention.

Damage to cells from the human nasal mucosa resulting from saline perfusions which were tested in the course of studies on vehicles used for plasma expanders (Pomerat and Overman[1]) led to a demonstration by Shambaugh[2] of the injurious properties of this solution to epithelial cells cultivated from the organ of Corti and from the middle ear mucosa of the cat. In both of these studies it could be shown that little damage followed contact with Gey's balanced salt solution. It is not surprising that a solution containing only two ions, sodium and chloride, should fail to meet the requirements of cells which normally are bathed by fluid containing a variety of organic and inorganic substances. The purpose of this study was to check on the vulnerability of cells from human and rabbit eyes to saline. Cells from the conjunctiva and cornea were used and the effects of several electrolyte solutions were compared.

MATERIALS AND METHODS

Cellular responses to environmental changes under controllable conditions have formed a major experimental outlook in this laboratory. The use of double time-lapse cine camera units, perfusion chambers, and the technique of film analysis have been described in several publications (Pomerat,[3] Pomerat, Lefeber, and Smith,[4] Pomerat and Smith,[5] Pomerat and Overman,[1] Pomerat, Kent, and Logie[6]).

Tissues employed in this study were human conjunctiva and rabbit cornea. Cells from the anterior and posterior surfaces of the rabbit cornea were examined separately. The human conjunctiva was obtained from patients undergoing cataract surgery. Corneal tissue was obtained from adult white rabbits. A layer approximately 0.1-mm. thick was dissected from the anterior and posterior surfaces. More than 125 explants were employed during the course of this study.

Explants of tissue measuring approximately 1.0 mm. on a side were washed with Gey's balanced salt solution (BSS) and embedded in a clot consisting of equal parts of heparinized cockerel plasma and extract from eight-day chick embryos on cover slips measuring 12 × 50 mm. When the clot was firmly set, 2.0 ml. of a fluid nutrient consisting of 50-percent human ascitic fluid of malignant origin, 45-percent Gey's BSS, and 5.0-percent chick embryonic extract to which 1,000 units/ml. of penicillin had been added was used as a feeding solution. These conditions have proven useful for the cultivation of various human eye tissues (Pomerat and Littlejohn[7]).

Solutions employed in these experiments were Gey's BSS, saline, and two commercially prepared electrolyte solutions. A comparison of the constituents is given in Table

* From the Department of Ophthalmology and the Tissue Culture Laboratory, Department of Anatomy, University of Texas Medical Branch. Aided by a grant from the National Society for the Prevention of Blindness, July 26, 1957.

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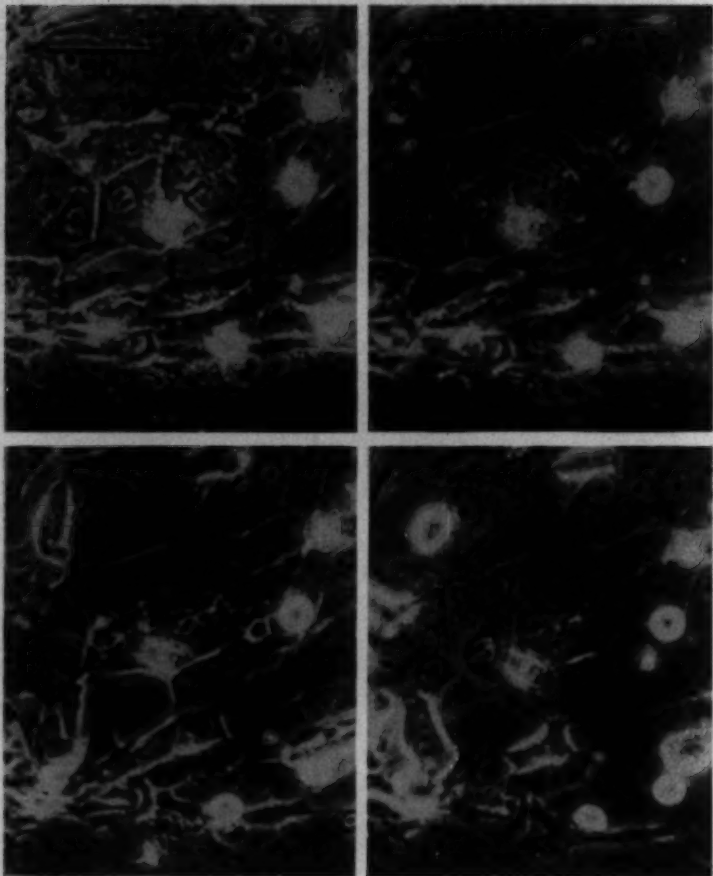

Plate 1* (Harper and Pomerat). *Selected film frames in a typical perfusion chamber experiment using a 5-day culture of human conjunctiva.*

1. In nutrient media.
2. After exposure to Gey's BSS for about one hour.
3. After saline had been present for about one hour. Little morphologic change was produced by this solution.
4. After nutrient fluid had been re-perfused for about one hour.

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* The scale of magnification for all figures is shown in the left upper area of Figure 1.

EXPLANATION OF PLATE 2 →

1. In nutrient media.
2. After one hour in Gey's BSS.
3. After one hour in Solution B. The field had been shifted slightly.
4. After one hour in Solution A.
5. Two minutes after the addition of saline. The cells showed retraction of their membranes.
6. Three minutes later there was a further disturbance in their appearance.
7. After one hour in saline the cells show further evidence of injury.
8. Sixteen hours after the re-perfusion with nutrient fluid. The cells remained individualized and did not reform an epithelial sheet.

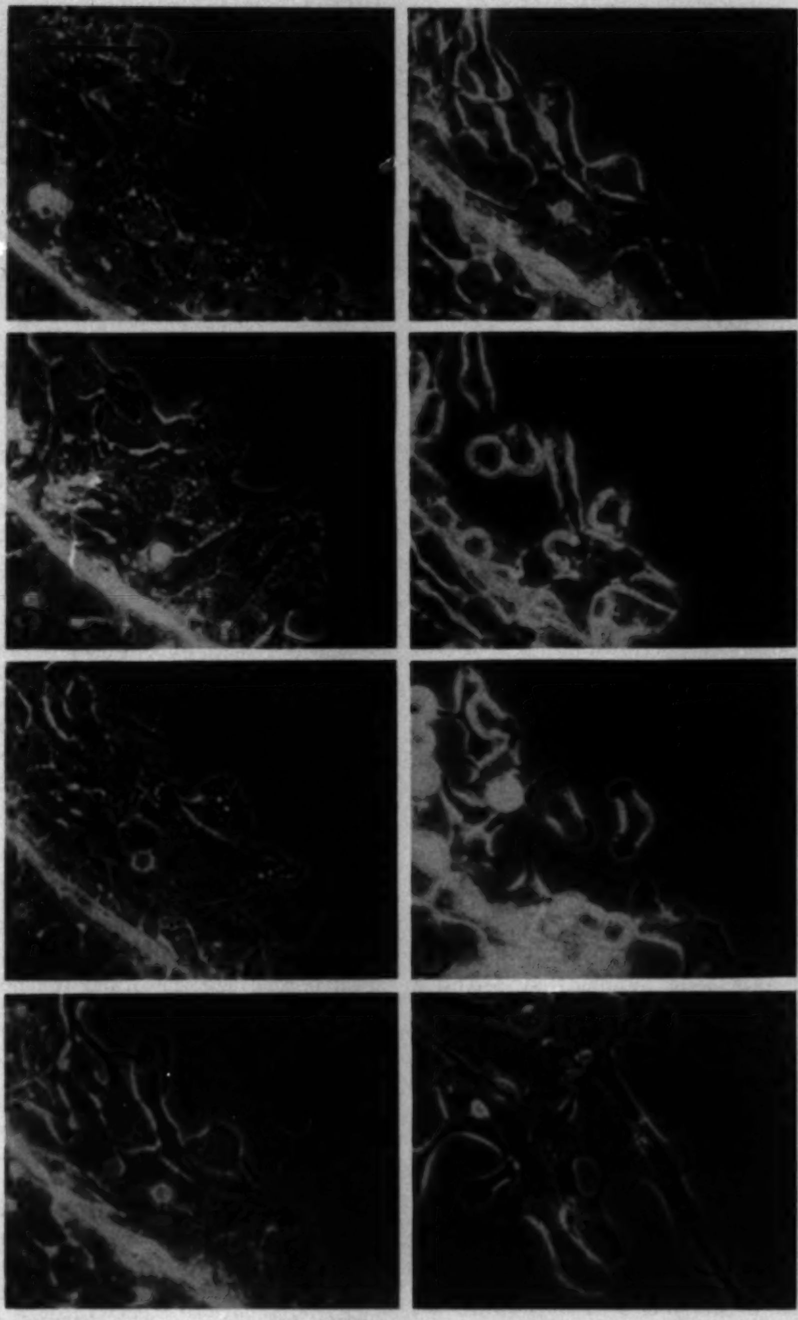

Plate 2* (Harper and Pomerat). *Selected film frames in a typical perfusion chamber experiment using a 6-day culture of rabbit corneal epithelium.*
(See facing page for explanation of figures)

* The scale of magnification for all figures is shown in the left upper area of Figure 1.

TABLE 1

CONSTITUENTS OF GEY'S BALANCED SALT SOLUTION AND TWO COMMERCIALLY PREPARED ELECTROLYTE SOLUTIONS

	Gey's BSS	Solution A*	Solution B†
	(gm./1000 cc.)		
NaCl	8.0	4.9	8.0
KCl	0.38	0.75	0.4
$NaHCO_3$	0.25		
$CaCl_2$	0.13	0.36	
$MgCl_2 \cdot 6H_2O$	0.21	0.30	
$Na_2HPO_4 \cdot 12H_2O$	0.30		
$Na_2HPO_4 \cdot 7H_2O$			0.0875
KH_2PO_4	0.025		0.0625
$MgSO_4 \cdot 7H_2O$			0.2
Glucose	1.0		1.0
NaAcetate		3.9	
NaCitrate		1.7	
pH	7.36	7.16	6.37

* Mead Johnson and Co.
† Baxter Laboratories, Inc.

1. Solution A was supplied by Mead Johnson and Co. and Solution B by Baxter Laboratories, Inc.

The fluids were perfused for one hour each in the following order:

1. The nutrient media in which the cells were grown.
2. Gey's BSS.
3. Solution B.
4. Solution A.
5. Normal saline.

Nutrient media was then reperfused and allowed to remain in contact with the cells overnight.

Cultures* were examined in perfusion chambers with phase contrast cine microscopy. Cell activities were followed through the periscope view finder of the camera while experiments were in progress. More than 2,000 feet of 16-mm. records were made with time-lapse technique. The optics employed consisted of an 8.0-mm. dark-phase objective without the use of an ocular.

TABLE 2

PERFUSION EXPERIMENT ON HUMAN CONJUNCTIVA

Plate I Figure	Solution Perfused	Time in Solution
1	Fresh supernatant	57 min.
2	Gey's BSS	58 min.
3	Saline	58 min.
4	Fresh supernatant	1 hr. and 20 min.

Total time of experiment was 4 hr. and 40 min.

RESULTS

A. EFFECT OF SALINE ON EPITHELIUM FROM THE HUMAN CONJUNCTIVA

Cells from the conjunctiva showed little change when exposed successively to Gey's BSS, Solution A, Solution B, and saline. Table 2 presents the protocol of a typical experiment and includes references to the figure illustrations of film frames selected approximately one hour after the fluids were brought in contact with five-day cultures of human conjunctiva (E. R. UH #4190-M).

B. EFFECT OF SALINE ON CELLS OBTAINED FROM ANTERIOR SURFACE OF RABBIT CORNEA

While little effect was produced by any of the balanced electrolyte solutions, following the addition of saline there was a disruption of the intercellular boundaries and the cells became individualized. However, with the return of the nutrient media, the elements again became aggregated and resumed the appearance of an epithelial sheet (table 3).

C. EFFECT OF SALINE ON CELLS OBTAINED FROM POSTERIOR SURFACE OF RABBIT CORNEA

Explants from the posterior surface of cornea showed two types of outgrowth. Some explants yielded cells which appeared similar to fibroblasts whereas excellent epithelial sheets were obtained from others. Possibly the spindle cells were derived from the stroma and the epithelial elements represented outgrowth from the endothelial layer.

* Cultures were prepared with the aid of Mrs. Walther Hild and Mr. Charles Raiborn. Acknowledgement is made to Messrs. George Lefeber and Earl Pitsinger for indispensable aid in the preparation of cine records and their analysis.

TABLE 3

PERFUSION EXPERIMENT ON RABBIT CORNEAL EPITHELIUM

Plate II Figure	Solution Perfused	Time in Solution
1	Fresh supernatant	58 min.
2	Gey's BSS	58 min.
3	Solution B	58 min.
4	Solution A	58 min.
5	Saline	2 min.
6	Saline	5 min.
7	Saline	58 min.
8	Fresh supernatant	15 hr. & 45 min.

Total time of experiment was 22 hr. and 43 min.

TABLE 5

EFFECT OF GEY'S BALANCED SALT SOLUTION (BSS) IN COMPARISON WITH SALINE FOLLOWING IRRIGATION INTO ANTERIOR CHAMBER OF RABBIT'S EYE

Rabbit	Number of Days Before Cornea Cleared		Difference in Clearing Time (da.)
	Saline	Gey's BSS	
A	7	2	5
B	4	2	2
C	4	2	2
D	7	3	4
E	7	3	4
F	7	4	3

The introduction of saline showed a definite effect on both of these cell systems. Cellular activity was disturbed with the cessation of intracellular granule movement. The undulating movements of the cell membrane were arrested with a retraction of cytoplasmic processes. When Gey's BSS was added these changes were intensified and further damage occurred. With the return of the nutrient fluid some cells survived but many were irreversibly damaged (table 4).

In order to determine if these findings might be of clinical significance the anterior chambers of 18 mature rabbits were irrigated with the test solutions under conditions designed to parallel surgical practice. Saline was introduced into the right eye and one of the balanced electrolyte solutions into the left anterior chamber of six animals. Each animal was sedated with intraperitoneal Nembutal. Local anesthesia was obtained with 0.5 cc. of two-percent Procaine retrobulbarly and 0.5-percent Pontocaine was applied topically. A corneal incision was made with a keratome and the anterior chamber irrigated during a four-minute period with 20 cc. of the appropriate solution.

In all experiments the side on which saline was used showed a more opaque cornea within 24 hours than the fellow eye. Although the eyes irrigated with the balanced electrolyte solutions showed a slight haze, this cleared one to seven days sooner than those on which saline was used (tables 5, 6, and 7).

CONCLUSION

In the light of these experiments it would seem that in the surgical care of delicate tissues of the eye, such as the corneal endothelium, it might be advisable to employ balanced salt solutions which have been proven

TABLE 4

PERFUSION EXPERIMENT ON RABBIT CORNEAL ENDOTHELIUM

Plate III Figure	Solution Perfused	Time in Solution
1	Fresh supernatant	58 min.
2	Gey's BSS	58 min.
3	Solution B	57 min.
4	Solution A	58 min.
5	Saline	2 min.
6	Saline	5 min.
7	Saline	58 min.
8	Fresh supernatant	15 hr. & 47 min.

Total time of experiment was 22 hr. and 43 min.

TABLE 6

EFFECT OF SOLUTION A IN COMPARISON WITH SALINE FOLLOWING IRRIGATION INTO ANTERIOR CHAMBER OF RABBIT'S EYE

Rabbit	Number of Days Before Cornea Cleared		Difference in Clearing Time (da.)
	Saline	Solution A	
G	2	1	1
H	7	2	5
I	6	2	4
J	6	3	3
K	6	2	4
L	5	1	4

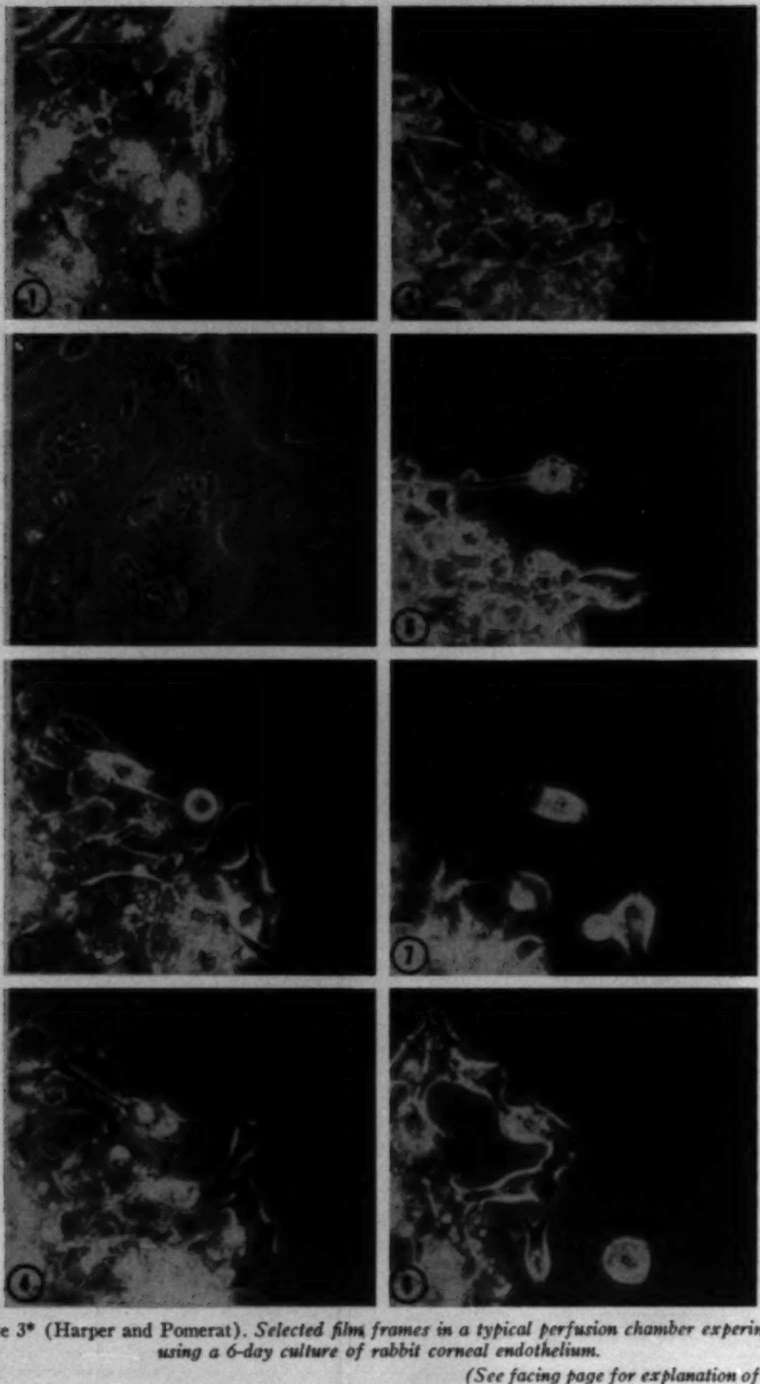

Plate 3* (Harper and Pomerat). *Selected film frames in a typical perfusion chamber experiment using a 6-day culture of rabbit corneal endothelium.*

(See facing page for explanation of figures)

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* The scale of magnification for all figures is shown in the left upper area of Figure 1.

TABLE 7

EFFECT OF SOLUTION B IN COMPARISON WITH SALINE FOLLOWING IRRIGATION INTO ANTERIOR CHAMBER OF RABBIT'S EYE

Rabbit	Number of Days Before Cornea Cleared		Difference in Clearing Time (da.)
	Saline	Solution B	
M	6	2	4
N	6	3	3
O	11	4	7
P	6	4	2
Q	10	4	6
R	7	2	5

safe for the management of tissue cells cultivated in vitro. The two commercial preparations which were tested compared favorably with Gey's balanced salt solution.

SUMMARY

The effect of various electrolyte mixtures was studied on cultures of cells from rabbit cornea and human conjunctiva with perfusion chamber technique using phase contrast, time-lapse cinematography. Gey's BSS and two commercially prepared electrolyte solutions showed little deleterious effects on any of the tissues. Saline was relatively innocuous for human conjunctiva and rabbit corneal epithelium but was definitely damaging to cells from the posterior surface of the cornea.

The advisability of employing commercially available physiologic solutions for washing delicate structures such as those of the eye is suggested.

University of Texas Medical Branch.

REFERENCES

1. Pomerat, C. M., and Overman, R. R.: Electrolytes and plasma expanders: I. Reaction of human cells in perfusion chambers with phase contrast, time-lapse cine records. Ztschr. Zellforsch., **45**:2-17, 1956.

2. Shambaugh, G. L.: Some of the technical problems in the surgery of otosclerosis. J. Internat. Coll. Surg., **25**:772-776 (June) 1956.

3. Pomerat, C. M.: Living human histology. XV° Cong. Soc. Internat. de Chirurgie, Lisbon, 1953, 1954, pp. 236-256.

4. Pomerat, C. M., Lefeber, C. G., and Smith, McD.: Quantitative cine analysis of cell organoid activity. Ann. N.Y. Acad. Sci. **58**:1311-1321, 1954.

5. Pomerat, C. M., and Smith, McD.: Moving pictures of body cells at work. Research Reviews, ONR, p. 17-21 (August) 1954.

6. Pomerat, C. M., Kent, S. P., and Logie, L. C.: Irradiation of cells in tissue culture. II. Cinematographic analyses of cell enlargement and mitotic activity following gamma irradiation at 2,000 r and 4,000 r. Ztschr. Zellforsch., **47**:175-197, 1957.

7. Pomerat, C. M., and Littlejohn, L., Jr.: Observations on tissue culture of the human eye. Southern M. J., **49**:230-237 (Mar.) 1956.

DISCUSSION

DR. MARGUERITE A. CONSTANT (St. Louis): This interesting work points up once again the question of just what is physiologic for cells. In this instance, so-called physiologic saline immediately causes disruption and damage to the endothelial cells, has no effect on conjunctival cells, and produces reversible damage to epithelial cells.

Some years ago Friedenwald showed that calcium was essential to maintaining the integrity of the ciliary body. It was noted in the present studies

←

EXPLANATION OF PLATE 3

1. In nutrient media.
2. After one hour in Gey's BSS.
3. After one hour in Solution B.
4. After one hour in Solution A.
5. Two minutes after the addition of saline. The cells showed retraction of their cell membranes.
6. Further retraction and individualization of the cells was noted after five minutes in saline.
7. After about one hour in saline no further changes were recorded.
8. Sixteen hours after the addition of nutrient fluid. Note the reaggregation of the cells forming an epithelial sheet.

that the three balanced solutions used contained either calcium or magnesium. It was also noted that the effect of saline was checked primarily after the cells had been incubated in salt solutions or, in the one instance in the paper, the salt solution was followed by Gey's balanced salt solution which produced further damage, and this damage was reversible.

As was noted on the first chart, the pH of these commercial solutions varied considerably, from 0.2 to 1.0 pH unit. Consequently, I should like to ask three questions pertaining to this aspect of the work.

One, which Dr. Harper has noted and which I think he should record because it is not in his paper, is this: What is the pH of the saline solutions that he used?

Secondly, it would be of interest to know whether or not the investigators studied the effect of saline immediately after nutrient media and replacing immediately with nutrient media, rather than superimposing the effect of the salt solutions on the cells.

Thirdly, whether they have investigated if the presence of calcium or magnesium is important and would make physiologic saline more physiologic for the endothelial cells.

The second portion of this work concerns data obtained from rabbits in vivo. These data support a recommendation that more balanced salt solutions should be used to irrigate the anterior chamber of humans. Theoretically such a recommendation is to be championed. Practically there may be objections, and I leave this aspect to those more familiar with the practical aspects of the operating room.

Although frequently one can extrapolate from animal data to humans, there may be a caution in this regard, for the rabbit is notorious in the lack of stability of the physiologic status quo of anterior ocular tissues. It is my understanding that the human eye is much more stable. For instance, it is less affected by radiation; it is less affected by paracentesis procedures, and so on. It was my impression from our perfusion studies in situ in humans, using saline for 30 minutes or more, that there was no untoward effect on the cornea. However, these were acute studies.

Therefore, I should like to ask the authors if they have had the opportunity to investigate the effects of balanced salt solutions on the endothelium of human eyes.

Not emphasized in this report is the admirable feat of culturing adult ocular tissues as a routine procedure, with the cells maintaining their identity. I should like to congratulate these workers on the development of this basic work and the beautiful cinematography shown here today.

Dr. John Y. Harper, Jr. (Galveston): The first question, on the effect of saline when using it immediately following the nutrient medium: That, of course, was done initially, and gave the effects which have been noted here. However, the difficulty in doing the experiment in this manner is that, once the saline has been used and the effects noted on the cells, the preparation is no longer satisfactory for using other perfusates. The reason why the saline was put last was because we wanted to compare the balanced electrolyte solutions with the saline.

I do not have any information on the effects of antimony, calcium, and magnesium added to saline in order to prevent the breakdown of the intracellular boundaries. However, I thank Dr. Constant for that point, because it probably would be a very interesting line to follow. At present electrolyte solution experiments have not been undertaken in humans but are planned in order to compare the effects of electrolyte solutions and saline in the human eye. The pH of the saline was 6.8.

CORNEAL DEVELOPMENT*

II. Transparency changes during rapid hydration

Alfred J. Coulombre, Ph.D., and Jane L. Coulombre, B.S.
New Haven, Connecticut

Introduction

The factors most usually cited as affecting the transparency of the vertebrate cornea are: (1) the pattern of deposition of collagenous fibers in the stroma, (2) the state of relative turgescence of the stroma, and (3) the presence of stromal mucopolysaccharides. Studies of the adult cornea have yielded evidence for the involvement of each of these factors. Since, however, they operate simultaneously in the adult cornea, and are, in many instances, difficult to uncouple experimentally, it has been impossible to weigh the relative contribution of each, or to specify their modes of interaction.

* From the Department of Anatomy, Yale University School of Medicine. This investigation was supported by a research grant, B-870 (c), from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service.

Studies of the developing cornea have the advantage that specific factors come into operation, and important conditions come into being, at well defined points in embryologic time. This situation has been exploited in the rabbit embryo (Smelser and Ozanics, 1956) and in the chick embryo (van den Hooff, 1951; Coulombre, 1956, 1958). In chick embryos, experiments have established that: (1) the definitive pattern of stromal collagen fibers begins to form on about the eighth day and is well established by the 14th, (2) the cornea begins to dehydrate on about the ninth day, and (3) metachromatic mucopolysaccharide is first detectable in the corneal stroma on the 14th day at which time the cornea begins to become transparent, to accelerate its rate of dehydration, and to become thinner.

This sequence of events is of interest for its direct bearing on the problem of corneal transparency. In addition, it affords a unique situation in which the factors and conditions of primary interest are uncoupled in developmental time for separate experimental attack.

Advantage was taken of this situation to study the hydration properties of the developing chick cornea experimentally at selected ages before, during, and after the deposition of metachromatic mucopolysaccharide in the stroma. In addition correlative changes in corneal transparency were recorded, for the same ages, under identical conditions of experimentally controlled hydration. The results are discussed in terms of their bearing on the physical basis of corneal transparency.

Material and Methods

The White Leghorn chick embryos used were incubated at 37°C.± 0.5°C. in a forced draft incubator. All ages are given as days from the onset of incubation.

The data are confined to four days (9th, 13th, 17th, and 21st), which span the period of greatest interest. The first series of measurements follows corneal hydration under experimental manipulation. The second records transparency changes under these same conditions.

Water uptake was determined under conditions of rapid hydration in distilled water. As in adult corneas (Kinsey and Cogan, 1942), the rate of water uptake from distilled water by chick embryonic corneas is initially rapid but approaches zero (equilibrium) within about 60 minutes at all the ages investigated. As a consequence, the data necessary to relate transparency to hydration were collected during the first hour following corneal immersion. This contrasts with experimental time intervals of many hours or even days used in most studies.

Corneas were excised from freshly dissected eyes so that a ring of scleral tissue remained attached at the limbus. At different time intervals following immersion, specimens were removed to a glass plate, the cornea was quickly freed of surrounding sclera, blotted on filter paper, and weighed in an aluminum tare to the nearest 0.01 mg. on a torsion balance. After drying to constant weight at 110°C. the specimen was reweighed. The cornea separates easily along a line at its limbus allowing precise segregation of corneal tissue and elimination of all scleral components. Water content was calculated as weight of water per unit dry weight. The values so obtained are referred to below as *specific hydration*. Ten specimens were used to determine the wet weight, dry weight, and specific hydration for each interval following immersion.

Transparency changes during hydration were followed photometrically under conditions of continuous graphic recording. Isolated corneas surrounded by a scleral ring were immersed in distilled water in a suitable container and centered in a small columnated beam of incandescent light. Emergent light impinged on a photocell whose output was used to drive a continuous recorder of high sensitivity and linear response over the range of intensities employed. The recorder was adjusted to record percent transmittance directly as a function of time. Continuous re-

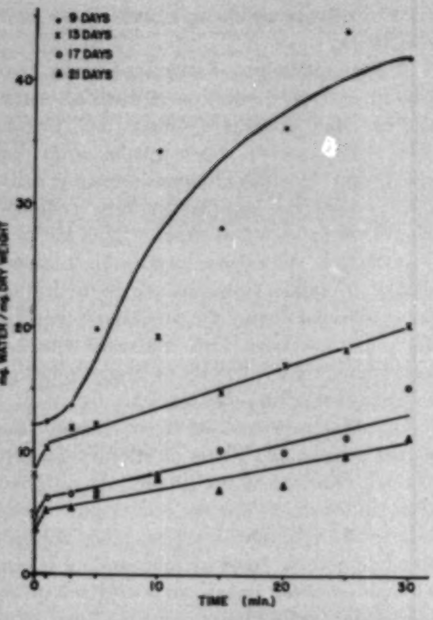

Fig. 1 (Coulombre and Coulombre). Specific hydration (mg. water/mg. dry weight) as a function of time, at four ages, for corneas immersed in distilled water at zero time. Each point on each curve represents the mean of measurements on 10 different corneas, a total of 360 corneas.

cordings of one-half hour duration each were made on 10 corneas at each of the ages studied. Mean curves were derived at each age by averaging selected points on the individual recordings.

Right and left eyes showed no significant differences for either type of data.

Results

Hydration

The specific hydration of untreated chick embryo corneas decreases from about 12 mg. water/mg. dry weight at nine days to about 3.5 mg. water/mg. dry weight at 21 days (Coulombre, 1958). Following immersion in distilled water, corneas of all ages imbibe water (fig. 1). Contrary to expectation, however, the younger corneas absorb relatively more water per unit dry weight than older corneas, throughout the experimental period. The curves of specific hydration during immersion not only fail to converge as equilibrium is approached, but they continue to diverge. This fact indicates that even under conditions of maximal hydration, there is an inverse relation between specific hydration and age. As the cornea matures each unit of its dry weight loses some of its absolute ability to imbibe and hold water. A recalculation of the data to express specific hydration as percent of the 21-day values and as a function of age illustrates this progressive loss (fig. 2).

Tranparency

It is well known that an increase in corneal water content above normal results in some degree of loss of transparency. It remains to express transparency as a function

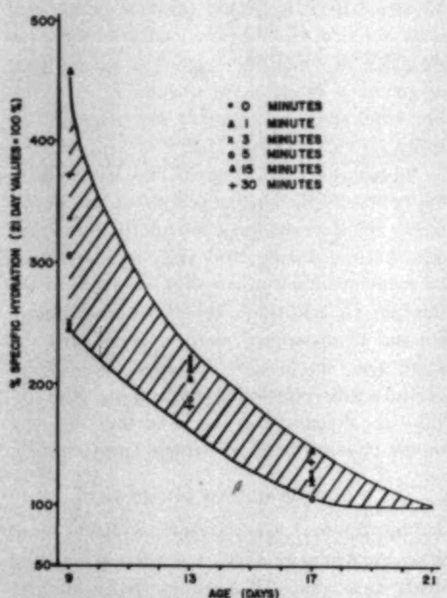

Fig. 2 (Coulombre and Coulombre). The data represented in Figure 1 are recalculated and replotted here to represent specific hydration as percent of the 21-day values, and as a function of age. There is a progressive decrease in specific hydration with age at all levels of hydration, even as equilibrium with distilled water is approached.

of corneal hydration. To make this possible we used continuous recording of changes in transparency from the moment the cornea begins to hydrate in distilled water. This reveals a highly consistent, but complex, pattern of change in transparency (fig. 3). This pattern consists of successive phases of increase and decreases in transparency. The physical basis of these fluctuations is under study and will not be considered here. For our present purposes, however, the following points should be noted. While the sequence of transparency fluctuations is identical for all of the ages investigated, the rapidity with which they occur, and their magnitude, increase with the age of the cornea.

The most remarkable feature is that the greatest decrease in transparency occurs during a relatively brief period following immersion of the cornea in distilled water. Thus, the 21-day cornea, which most nearly approximates the adult condition, undergoes nearly all of its decrease in transmittance during the first six minutes of immersion. Yet during this period the cornea has taken up only a fraction of the total amount of water it will absorb at equilibrium. A relatively small change in specific hydration is responsible for a dramatic loss in light transmittance (fig. 4). That this loss may occur in two steps is indicated by the dotted line in Figure 3. The first step occurs so rapidly, however, that it appears on only occasional records.

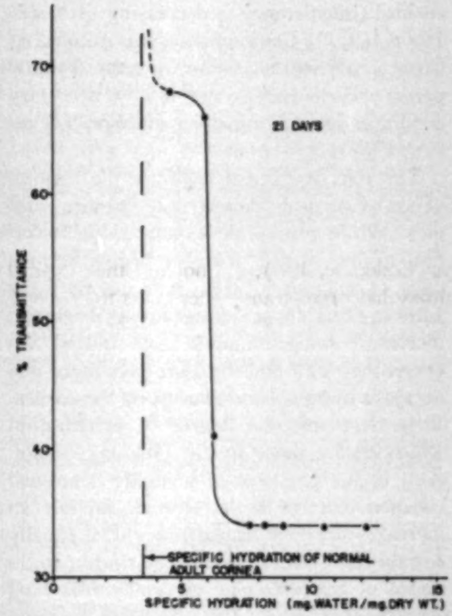

Fig. 4 (Coulombre and Coulombre). Transmittance of the 21-day cornea is rendered as a function of specific hydration. The plot was made with data drawn from the studies of hydration and transparency change under comparable conditions.

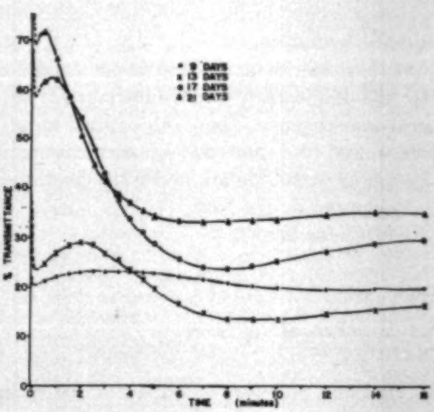

Fig. 3 (Coulombre and Coulombre). Fluctuations of corneal transparency during immersion in distilled water are plotted as functions of time for four ages. Each curve represents the mean of determinations on 10 corneas under conditions of continuous recording.

Comment

A synthesis of data from the present study with previously reported results permits a partial evaluation of the role of the three factors listed in the introduction as affecting corneal transparency. While this discussion is limited to data on the chick embryo it is probable that many points are applicable to other species (Smelser and Ozanics, 1956, on the rabbit).

The definitive collagenous pattern of the corneal stroma is established between the eighth and the 14th day. During this interval

corneal transparency is decreasing (van den Hooff, 1951). Consequently, this patterning is not a sufficient condition for corneal transparency. Whether or not it is a necessary condition remains an open question (Maurice, 1957).

The role of corneal water in the maintenance of corneal transparency is more complex. While normal, developmental dehydration of the cornea probably begins at nine days (Coulombre, 1958), it is not until the 14th day that corneal transparency begins to increase toward the adult level. Indeed, between the ninth and the 14th days there is a decrease in light transmission by the cornea. It is clear that the degree of dehydration which occurs prior to the 14th day cannot, even in the presence of a highly developed collagen pattern in the stroma, support an increase in corneal transparency. It is equally certain that the development and maintenance of transparency, especially following the pivotal 14th day, requires optimal stromal turgescence. The dramatic decrease in corneal transparency which accompanies even a slight increase in specific hydration not only indicates a role for stromal water but underscores the fact that transparency is not a simple function of hydration. The complex variations in transparency during the course of rapid hydration are as yet unexplained. It is possible, however, that these fluctuations may provide a key to changes during hydration in corneal components other than the stroma (epithelium, endothelium, and membranes) which could contribute to the experimentally induced loss in transparency. Finally, the absolute loss in ability to absorb water as development proceeds invites attention to the factors which underlie this important phenomenon.

A more central role is suggested for the metachromatic mucopolysaccharide of the stroma. It is first detected in the stroma on the 14th day (Coulombre, 1958). Once it appears, the last of the conditions necessary for the development of transparency seems to be fulfilled and the cornea begins a rapid increase in transparency that achieves the adult level at 19 days.

Summary

The following conclusions apply to the developing cornea of the chick embryo:

1. The specific hydration of the untreated embryonic cornea decreases with age.

2. The capacity of the cornea to absorb water under conditions of rapid hydration is greater at younger ages. This age differential is maintained even when corneas approach hydration equilibrium with distilled water.

3. Transparency is a multiphasic function of corneal hydration. Under conditions of rapid hydration the bulk of the loss of transparency occurs during a very small change in specific hydration.

4. No one factor is a sufficient condition for the development and maintenance of corneal transparency. That both optimal turgescence and the presence of metachromatic mucopolysaccharide are necessary conditions is suggested by the data.

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References

Coulombre, A. J.: Development of corneal transparency. Anat. Rec., **124**:278, 1956.

Coulombre, A. J., and Coulombre, J. L.: Corneal development. I. Corneal transparency. Cell. & Comp. Physiol., in press, 1958.

Kinsey, V. E., and Cogan, D. G.: The cornea. III. Hydration properties of excised corneal pieces. Arch. Ophth., **28**:272-284, 1942.

Maurice, D. M.: The structure and transparency of the cornea. J. Physiol., **136**:263-286, 1957.

Smelser, G. K., and Ozanics, V.: Studies on the differentiation of the cornea and sclera of the rabbit. Anat. Rec., **124**:362, 1956.

van den Hooff, A.: De Doorzichtigheid van de Cornea. Ned Tijdschr. Geneeskunde, **95**:34-36, 1951.

Discussion

Dr. George K. Smelser (New York): We are very fortunate to have these studies of Coulombre of the development of the chick eye, because they bring out interesting and important differences from the observations of the development of the mammalian eye. It has been shown that the chick cornea becomes dehydrated during development. In the early embryo various organs contain a good deal more water than their counterpart in the adult or even in the older fetus. Is the decrease in water content of the cornea different in rate or time pattern from that of a non-transparent connective tissue such as the sclera? The dehydration curves of the cornea and sclera during the development of the rabbit eye are different.

In the early stages of development it was pointed out that the cornea is not very transparent and that fine collagen fibrils are present. Is it not possible that in these early stages the fibers may be morphologically well arranged, as seen in the light microscope, yet not possess the precise spatial arrangement in a lattice as demanded by Maurice's concept of a corneal transparency? According to this theory, transparency depends upon such a precise relationship. It may very well be that in the embryo the fibers had not yet attained the required pattern.

The experiments of Coulombre on the hydrophilia appear on first inspection to differ from those made in the rabbit (Anat. Rec., **124**:362, 1956) in which corneal hydrophilia was found to increase as the embryo developed. However, it is very possible that no difference exists. The hydrophilia was calculated, in the present experiments, on the basis of dry corneal tissue, whereas the water imbibition curves of the embryonic rabbit corneas referred to were based on fresh weight. As the cornea develops the major increase in dry weight may be of collagen, which is much less hydrophilic than the ground substance. The more hydrophilic young embryos reported here probably contained a higher proportion of mucoid than collagen, hence the greater hydrophilia in the younger than in the later stages. It would be interesting to have the author's comment on this suggestion. Another point raised by the present paper is shown in the graphs presented by Dr. Coulombre which showed an extremely rapid decrease in transparency when the cornea was immersed for a very short time in distilled water. Is it not possible that this phenomenon may also be explained on the basis of the lattice theory of corneal transparency? Possibly the first invasion by water between the corneal fibrils tended to disturb their neat arrangement. As soon as the fibrils are displaced the normal lattice no longer exists and, according to Maurice, transparency should decrease. As the cornea continues to swell by the imbibition of larger quantities of water, the spatial arrangement of the fibrils would be disturbed further, but the major shift in transparency would have already taken place.

I am very grateful for the opportunity to discuss this paper which has dealt with so many important aspects of corneal differentiation and particularly so because now investigations on the development of the eye of two unrelated vertebrates so that similarities and differences in their development can be studied.

Dr. Albert M. Potts (Cleveland): This work is of particular interest to us because we are in the middle of a study on corneal transparency versus corneal hydration at the present time. There is one small point that occurred to us the hard way because we suspected at last that it might be suggested as a control.

We again were concerned about relationships between amount of water imbibed and transparency, and found that our conclusions had to be completely revised when we stripped epithelium from cornea. One can't talk about cornea as a single tissue. Whereas the epithelium represents only a relatively small part of the mass, in the experiments we were doing with isolated beef corneas, the epithelium contributed some four or five times as much to the loss of transparency, to the light absorption, as did the stroma under our particular experimental conditions.

This is something that certainly has to be controlled whenever one is doing such a measurement.

Dr. Alfred J. Coulombre (closing): I know we are overdue for a recess, so I will attempt to make this brief. Thank you, Dr. Smelser, for your comments, which were, as usual, most interesting and pertinent.

In the chick embryo as in the rabbit embryo we do, indeed, find an initial phase of slow corneal dehydration followed later in development by a phase of rapid dehydration, the second commencing at a time when we first detect metachromatic mucopolysaccharide histochemically in the stroma.

In reference to the physical basis of corneal transparency, the rapid change in transparency with a small degree of hydration seems more consistent with the hypothesis of Maurice because of its rapidity alone, than with other hypotheses now current.

However, as Dr. Potts pointed out, we have a very complex situation in dealing with the entire cornea. It is necessary for us now to dissociate the contributions of each of the layers of the cornea to the composite curves that we have presented for both hydration and for transparency.

An intriguing possibility that should be viewed against the background of overconcern with the stroma is that the epithelium itself may contribute an enormous amount not just to hydration but to the opacity which develops during hydration.

OBSERVATIONS ON THE SITE AND MECHANISMS OF ANTIGEN-ANTIBODY INTERACTION IN ANAPHYLACTIC HYPERSENSITIVITY*

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Previous work from this laboratory has been concerned primarily with the dynamics of antigen-antibody interaction and the relationship of this process to the tissue lesions in hypersensitivity of the serum sickness type. These investigations integrated with the work of others may be briefly summarized.

Studies by Hawn and Janeway[1] and ourselves[2, 3] have demonstrated that the lesions of serum sickness appear as an animal begins to form antibody while antigen is still present in the circulation and tissue fluids. With continued production of antibody, antigen is eliminated, free antibody appears in the circulation, and the organic manifestations of serum sickness resolve. Since antigen is present in the blood during the early antibody response, it was suggested by us some years ago[2, 3] that the tissue lesions might arise secondary to the transport of antibody by antigen in the form of soluble antigen-antibody complexes. In the intervening years, Sternberger, et al.,[4] and Weigle[5] have demonstrated antigen-antibody complexes in the circulation. Mellors, et al.,[6] and Dixon, et al.,[7] employing fluorescent antigen and antibodies found antigen and gamma globulin localized in the tissue lesions of hypersensitivity. It is not known whether the localization of gamma globulin in the tissue represents specifically fixed antibody. Other work from our laboratory has indicated that soluble antigen-antibody complexes produced in vitro may elicit anaphylactic shock in guinea pigs, produce Arthus reactions in the skin, and induce glomerular and splenic lesions of the serum sickness type following infusion into normal rabbits.[8, 9] Presumably these reactions require complement.[10, 11] There is then considerable evidence that soluble antigen-antibody complexes exhibit significant biologic activity and that they may play a leading role in the development of allergic reactions.

It is clear that important strides have been made in an area which may be called the immunogenesis of immediate-type allergic reactions. Two of the most important problems which still demand clarification concern firstly, the mechanism by which antigen-antibody-complement complexes injure tissue, and, secondly, the site of tissue injury.

In regard to the precise biochemical mechanism through which antigen-antibody-complement interaction results in tissue injury little is known. Studies currently being carried on by others[12, 13] on the role of complement in the hemolysis of red blood cells could provide data which might contribute to an understanding of tissue damage in allergic reactions. Many workers believe that complement is concerned in the activation of an enzymatic system in the presence of reactive antigen and antibody.[12, 13]

In regard to the site of tissue injury in allergic reactions, numerous studies have been made. There seems little doubt that anaphylactic reactions are manifested in the vascular and connective tissue systems. Whether dâmage to the vascular system is primarily an expression of alteration in the connective tissue which is indissolubly associated with blood vessels or whether these changes are due to endothelial injury has never been resolved. Since marked alteration of the connective tissues is prominent in hypersensitivity, the first of these possibilities seems more

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acceptable and warrants further investigation.

Some years ago, Rich and Follis[14] failed to produce a typical hemorrhagic Arthus reaction in the rabbit cornea unless vascularization had been previously induced. They noted "slight edema, slight polymorphonuclear infiltration, and moderate swelling of the corneal fibers" 24 and 48 hours after injection of horse serum into the avascular cornea of sensitized animals. It would seem that these workers produced a true Arthus phenomenon in the vascularized corneas of sensitized animals. However, this might be expected under the conditions of the experiment since vascularization of the cornea in effect converts the cornea into a structure very similar to skin.

Since the cornea consists of almost pure connective tissue comprised of fibroblasts, collagen, and reticulum fibers, and amorphous "ground substance" far removed from a blood supply, and since there is so much experimental and pathologic evidence of connective tissue damage in hypersensitivity, it was deemed useful to employ this tissue in further studies of hypersensitivity, using quantitative immunologic methods to see whether connective tissue damage could indeed be related to antigen-antibody interaction in the absence of blood vessels. Recent work in our laboratory has disclosed that connective tissue change can be produced in the avascular cornea at the site of local antigen-antibody interaction. These experiments were of two designs. In one type, appropriate quantities of bovine serum albumin or bovine serum gamma globulin were injected into the avascular cornea of passively or actively sensitized rabbits. In the second type of experiment, bovine serum albumin and its corresponding antibody were simultaneously injected into different sites of the avascular corneas of normal rabbits. In all experiments, alterations in the connective tissue were observed which have been interpreted as those of degeneration or necrosis. Although these changes were accompanied by acute exudative inflammation, they did not appear to be dependent on leucocyte migration since similar changes were noted in animals which had been rendered leucopenic by nitrogen mustard injections. In each case, the connective tissue alteration occurred at the site of maximal precipitation of antigen-antibody complexes as demonstrated by fluor-marking of the injected antigen.

Following the injection of 0.1 ml. of fluor-labeled foreign protein into the center of the cornea of actively or passively sensitized animals, the limbus appeared grossly inflammed six to eight hours later. From 12 to 24 hours later, a grayish arc of opacification appeared in the periphery of the cornea. It was separated from the limbus by a clear area one to three mm. in width (fig. 1). Further observation for an additional 24 hours generally disclosed completion of the arc to form a concentric ring of opacification. Examination of such eyes under Wood's light in the living animal revealed

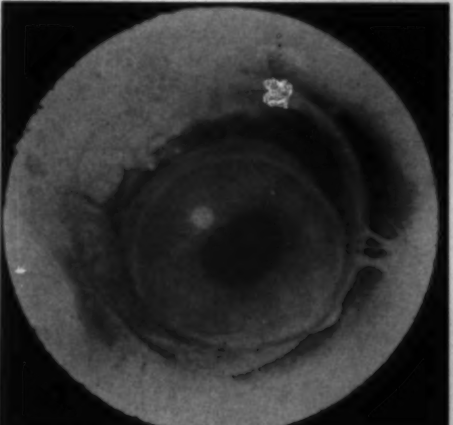

Fig. 1 (Germuth, et al.). Living eye in rabbit previously sensitized to bovine albumin. Twenty-four hours after injection of 1.0 mg. fluor-bovine albumin into center of cornea. Note opaque ring in periphery of cornea with clear cornea between ring and limbus. Dilatation and engorgement of limbal and bulbar conjunctival vessels with petechial hemorrhages. Under Wood's light the gray opaque ring presented bright green fluorescence. (The two bright spots are highlights.)

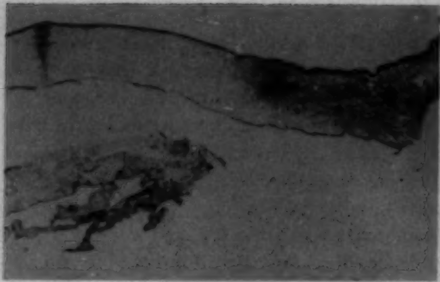

Fig. 2 (Germuth, et al.). Transverse section of peripheral portion of cornea and adjacent limbus. Rabbit previously sensitized with bovine albumin. Received injection of 1.0 mg. fluor-bovine serum albumin in center of cornea 30 hours before death. Hemorrhage and neutrophilic leukocytic infiltration around limbal vascular loops. Sharp transverse line of leukocytic infiltration and collagen degeneration distant from limbus. This line corresponds to a section through the opaque ring seen grossly. Note leukocytes migrating from limbus across clear corneal zone to line. No blood vessels are present in cornea.

sharp localization of antigen in the ring of opacification.

Histologic examination of corneas showing such rings (fig. 2) revealed a sharp line of concentrated leukocytes traversing the cornea from Bowman's to Descement's membrane. The leukocytes had evidently migrated from the limbal side as evidenced

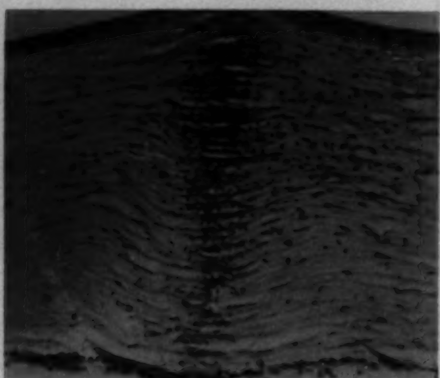

Fig. 3 (Germuth et al.). Moderate magnification of cornea to illustrate the histologic appearance of the opaque ring. Leukocytic infiltration and swelling and degeneration of corneal stroma.

Fig. 4 (Germuth et al.). Fluorescence preparation of lesion similar to that of Figure 3. The intense white line represents a bright green line of fluorescence under ultraviolet illumination due to fixation of fluor-antigen by antibody diffusing from limbus in a sensitized animal 30 hours after intracorneal injection 1.0 mg. fluor-bovine serum albumin. The line of fluorescence coincides precisely with the line of leucocyte infiltration and collagen degeneration. (The bright appearance of the cells is due to autofluorescence.)

by the presence of scattered leukocytes between the limbus and the sharp line of leukocytic infiltration. The limbal vessels were dilated and engorged and the loose connective tissue around them was infiltrated by leukocytes, red blood cells, and fibrin. Closer examination of the corneal line of leukocytes disclosed fragmentation of deeply eosinophilic swollen collagen fibers. The surrounding cells appeared pyknotic and their differentiation was not possible (fig. 3).

When corneal sections were examined with ultraviolet illumination the following typical changes were noted (fig. 4). There was maximal fluorescence along the corneal line of most intense leukocyte concentration and collagen fragmentation. No fluorescence was evident beyond this line toward the limbus except for autofluorescence of the granular leukocytes. The corneal tissue between the site of injection and the line showed diffuse pale greenish fluorescence indicative of antigen diffusing from the site of injection toward the periphery of the cornea. No

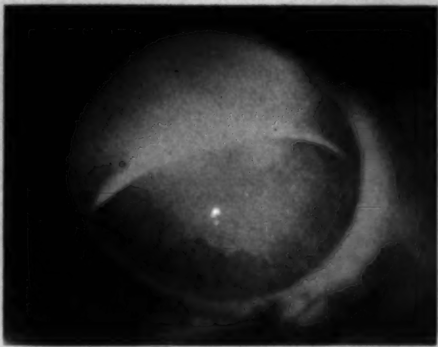

Fig. 5 (Germuth et al.). Line of opacification appearing between the separate site of injection of antigen and antibody in a normal rabbit cornea. Three days after injection of 0.8 mg. of antibody and 0.5 mg. of fluorescein-tagged bovine serum albumin.

Fig. 6 (Germuth, et al.). Histologic section of the line shown in Figure 5. Twenty-four hours after injection of normal cornae. The line is represented by an amorphous mass of deeply eosinophilic material which appears to separate the collagen fibers and which traverse the full thickness of the cornea at right angles to the surface. Leucocytic infiltration is mild at this stage.

blood vessels were found in the cornea.

When antigen and antibody were injected into separate sites in the normal cornea, a line of opacification appeared between the two sites of injection oriented at right angles to an imaginary line joining these (fig. 5). After 24 to 48 hours, the line was histologically represented by an amorphous mass of deeply eosinophilic material which appeared to separate the collagen fibers and which traversed the full thickness of the cornea at right angles to the surface (fig. 6). Leukocytic reaction at this stage was mild. This became more prominent with time so that by the seventh day the lesion was similar to that seen among the sensitized animals 48 hours after intracorneal injection of antigen. Fragmentation of the collagen fibers was not as evident in this experiment as it was in previously sensitized animals receiving intracorneal injection of antigen.

When the line of antigen-antibody precipitation in the corneas of nonsensitized animals was followed grossly over a period of days following the simultaneous injection of antigen and antibody, it was observed that the line progressed as an opaque front away from the antigen side toward the antibody side. This was readily demonstable by marking the cornea at the initial line of precipitation with India ink. Progressive development of the zone of opacification could be equated with antigen-antibody interaction as evidenced by gross examination under Wood's light and by fluorescence microscopy (fig. 7).

It is believed that these experiments demonstate that antigen-antibody interaction can produce immediate damage to the connective tissue stroma of the avascular rabbit cornea and that this damage is exquisitely localized

Fig. 7 (Germuth, et al.). Fluorescence preparation of lesion similar to Figure 6. The intense white line represents a bright green line of fluorescence under ultraviolet light. Migration of the line as described in the text has resulted in a double line of fluorescence.

at the site of interaction. Study of such lesions uncomplicated by the confusing effects and local participation of blood vessels as in the hemorrhagic Arthus phenomenon in the skin has obvious advantage not only for morphologic analysis but, more importantly, for chemical and enzymatic investigation.

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References

1. Hawn, C. van Z., and Janeway, C. A.: Histological and serological sequences in experimental hypersensitivity. J. Exper. Med., **85**:571, 1947.

2. Germuth, F. G., Jr.: A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type. J. Exper. Med., **97**:257, 1953.

3. Germuth, F. G., Jr., Pace, M. G., and Tippett, J. C.: The effect of sensitization to homologous and cross reactive antigen on the rate of antigen elimination and the development of allergic lesions. J. Exper. Med.. **101**:135, 1955.

4. Sternberger, L. A., Maltaner, F., and DeWeerdt, J.: Estimation of circulating antibody-antibody complex. J. Exper. Med., **98**:451, 1953.

5. Weigle, W. O.: The nature of antigen-antibody complexes found in rabbits during an immune response to bovine serum albumin. J. Exper. Med., **107**:653, 1958.

6. Mellors, R. C., Arias-Stella, J., Seigel, M., and Pressman, D.: Analytical pathology: II. Histopathologic demonstration of glomerular-localizing antibodies in experimental glomerulonephritis. Am. J. Path., **31**:687, 1955.

7. Dixon, F. J., Vazquez, J. J., Weigle, W. O., and Cochrane, C. G.: Pathogenesis of serum sickness. Arch. Path., **65**:18, 1958.

8. Germuth, F. G., Jr., and McKinnon, G. E.: Studies on the biological properties of antigen-antibody complexes: I. Anaphylactic shock induced by soluble antigen-antibody complexes in unsensitized normal guinea pigs. Bull. Johns Hopkins Hosp., **101**:13, 1957.

9. Germuth, F. G., Jr.: Experimental anaphylactic glomerulonephritis and human nephritis in Proc. Ninth Annual Conf. on the Nephrotic Syndrome. Edited by Metcoff, J.: 1957, p. 39.

10. Schwab, L., Moll, F. C., Hall, T., Brean, H., Kirk, M., Hawn, C. van Z., and Janeway, C. A.: Effect of inhibition of antibody formation by x-radiation or nitrogen mustards on the histologic and serologic sequences, and on the behavior of serum complement, following single large injections of foreign protein. J. Exper. Med., **91**:505, 1950.

11. Osler, A. G. Hawrisiak, M. M., Ovary, Z., Siqueira, M., and Bier, O. G.: Studies on the mechanism of hypersensitivity phenomena: II. The participation of complement in passive cutaneous anaphylaxis of the albino rat. J. Exper. Med., **106**:811, 1957.

12. Cushman, W. F., Becker, E. L., and Wirtz, G.: Concerning the mechanism of complement actvie. III. Inhibitors of complement activity. J. Immunol., **79**:80, 1957.

13. Ratnoff, G. O., and Lepow, I. H.: Some properties of an esterase derived from preparations of the first component of complement. J. Exper. Med., **106**:327, 1957.

14. Rich, A. R., and Follis, R. H.: Studies on the site of sensitivity in the Arthus phenomenon. Bull. Johns Hopkins Hosp., **66**:106, 1940.

Discussion

Phillips Thygeson (San Jose, California): The authors report recent work from their laboratory which indicates that connective tissue changes can be produced in the avascular cornea at the site of local antigen-antibody interaction. The changes noted were interpreted as degeneration or necrosis and were considered to be independent of leucocytic migration since similar changes were noted in animals rendered leucopenic by nitrogen mustard injections. In each case they noted that connective tissue alteration occurred at the site of maximal precipitation of antigen-antibody complexes as demonstrated by fluor-marking of the injected antigen.

In view of the acute exudative inflammation, which was a characteristic feature of the experiments, the elimination of leukocytic migration as a factor in the necrotic reaction seems to be of special importance.

The development of grayish areas of opacification in the peripheral corneas of sensitized animals after injection of foreign protein into the center of the cornea immediately calls to mind the phenomenon of marginal corneal infiltration and ring ulcer in staphylococcic blepharokeratoconjunctivitis in which staphylococciexotoxin is liberated in to the conjunctival sac in presumably sensitized animals. The resemblance to the precipitation ring that occurs in the agar plate precipitin technique is also striking. The authors' demonstration of sharp localization of antigen in the ring of opacification is most convincing and their histologic demonstration of fragmentation of eosinophilic swollen collagen

fibers leaves little doubt that damage occurred at the site of maximal antigen-antibody concentration.

The authors' experiments in which antigen and antibody were injected into separate sites in the normal cornea also calls to mind the agar precipitin technique. The similarity of the damage to collagen fibers at the line of opacification in the normal corneas as compared to the sensitized corneas is striking, and the delay in the development of this damage (seven days as compared to 48 hours) is understandable. Of special interest was the progression of the opacity away from the antigen site toward the antibody site and its relation to antigen-antibody interaction as evidenced by fluorescence microscopy.

This demonstration that antigen-antibody interaction can produce immediate damage to the connective tissue stroma is of far-reaching importance. The advantage of an avascular structure such as the cornea for immunologic studies is obvious, and it seems likely that chemists and immunologists will turn to this tissue more and more in the future. It is to be hoped that the authors will continue their important studies in this field. While they have well demonstrated the site of tissue injury much more remains to determine the exact mechanism by which antigen-antibody-complement complexes produce tissue damage.

Dr. Seymour P. Halbert (New York): These observations, of course, are extremely intriguing. I would like to ask the authors whether there is a possibility that the tissue effect that occurs in the cornea may be due merely to the physical presence of the antigen-antibody precipitate, and has nothing to do with the secondary factors which are thought to be involved in other hypersensitivity reactions. One might possibly study this by using animals in which the complement level has been decreased through some means or other.

Dr. Harold Falls (Ann Arbor): Dr. Thygeson has mentioned several clinical entities of hypersensitivity etiology which exhibit corneal manifestation. These infiltrations are usually just inside the limbus. I should like to add two additional clinical entities which have been postulated to have hypersensitivity causation and also exhibit corneal perilimbal infiltration: (1) Atopic eczema, reported by Dr. Hogan and his associates; (2) Cogan's syndrome—nonsyphilitic interstitial keratitis. Some authorities feel that this syndrome is a manifestation of periarteritis nodosa. Good evidence is available to support the concept that the latter is a hypersensitivity reaction.

Dr. Michael J. Hogan (San Francisco): I have seen a number of patients with identical lesions to the ring type of infiltrate which was seen in the initial slide, with many recurrences, each time with a complete ring infiltrate and each time a little greater distance into the cornea, the ring infiltrate always occurring just beyond the line of vascularization in the normal cornea itself.

Also, I wonder if the authors have seen the article in the last issue of the *British Journal of Ophthalmology,* in which identical lesions were produced by intracorneal and anterior chamber injections of heterologous sera. The one photograph shown in that article was of a typical ring infiltrate in the peripheral cornea.

Dr. C. E. Van Arnam (closing): In answer to Dr. Hogan, I have not seen that article, but the nature of the article and the references Dr. Falls and Dr. Thygeson have made to the resemblance of this experimentally produced ring to clinical cases prompts me to state that the original experiment was prompted because one of the authors, Dr. Pratt-Johnson actually observed clinically such a ring infiltrate in measles keratitis. It was one of the things he had been thinking about, and when the opportunity was presented to explore it he was actually early in the work.

The paper we have presented is in the nature of a preliminary report. Many of these things that have been mentioned have also come to our minds. It just seems that there is still a lot of work to be done and that there are a lot of possibilities of using this particular technique.

In reply to Dr. Halbert's question as to whether the physical presence of an antigen-antibody precipitate causes the damage, in contrast to the possibility that we have suggested, that it is due to some biologic activity, I am sure we do not have an answer. The senior author, Dr. Germuth, at the present time is working on the problem of complement and its presence in these lesions.

The next step in our work is to determine whether complement was present in the antigen-antibody complex in the cornea. I am not able to report anything on that at this time.

STUDIES ON THE CRYSTALLINE LENS*

VIII. A SYNTHETIC MEDIUM FOR LENS CULTURE AND THE EFFECTS OF VARIOUS CONSTITUENTS ON CELL DIVISION IN THE EPITHELIUM

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Detroit, Michigan

The object of this and earlier studies[1,2] has been to formulate a synthetic medium adequate to maintain lenses in a normal physiologic state. The number of cells undergoing mitosis in the epithelia of cultured lenses has been used as an index of adequacy of the medium to support growth of the lens.[2] A medium previously described[2] (KEI No. 1) did not maintain mitotic activity at the level found in lenses in the living animal nor at a level as high as could be obtained when rabbit aqueous humor was used as culture medium.

The present paper deals with studies directed toward an improvement of medium KEI No. 1 and includes determinations of the effects on mitosis of such agents as ascorbic acid, glutathione, cysteine, glutamine, triphosphopyridine nucleotide, some non-essential amino acids, inositol, and kinetin. The influence of the temperature and the redox potential of the medium on mitosis were also ascertained.

The result of this investigation is an improved medium, KEI No. 2, whose composition is given. Its temporary adequacy to support mitosis in the epithelium of the cultured lens is compared with its ability to keep the lens surviving, but not growing. This medium has already been employed in studies of the utilization of carbohydrates by the lens[3] and of the C^{14}-glucose metabolism of that organ.[4]

METHODS

Lenses of albino rabbits weighing between 1.5 and 2.5 kg. were cultured using an apparatus and technique described in detail earlier.[1,2] The animals were killed by air embolism, their eyes enucleated, and the lenses excised carefully. The average interval between the time of death of the animals and that of placing of the lens into the medium was about three minutes.

One lens from each animal was fixed immediately and served as control, while the other lens was cultured with the epithelial side up in five ml. of medium. A gas mixture consisting of seven-percent oxygen, five-percent carbon dioxide, and 88-percent nitrogen was passed continuously over the medium which was maintained at 37°C. and at a pH of 7.5. One hour was allowed for equilibration to insure proper pH and temperature at the start of the experiment. The longest period of culture without exchange of the medium was 24 hours, and sterile precautions were observed throughout.

Flat mounts of Feulgen-stained epithelia of cultured lenses and controls were prepared with the use of the method of Howard[5] as modified by von Sallmann.[6] The total number of epithelial cells in all phases of mitosis was determined in the cultured lens and compared with that in the uncultured contralateral lens on a percentage basis.

Determinations of the redox potentials of media were made with the use of a glass chamber device[7] which was kindly put at our disposal by Dr. Bernard Schwartz. Use of this apparatus made it possible to measure potentials without loss of carbon dioxide and thus under conditions of constant pH. A gold electrode was substituted for the platinum electrode and a calomel electrode served as a reference.

Analyses of glutathione were made by iodate titration in the usual manner. Glucose

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TABLE 1

EFFECTS OF VARIOUS COMPOUNDS ON MITOSIS IN EPITHELIA OF CULTURED LENSES

No. of Lenses	Hours of Culture	Medium	Mitosis in Vitro/in Vivo		Range
		KEI No. 1	%		%
9	9	KEI No. 1	56		22– 86
4	9	With six nucleosides omitted	61		42– 81
12	9	With alpha-ketoglutaric acid replaced by glutamine and TPN added (KEI No. 2)	86	σ16.7	46–124
		KEI No. 2			
4	9	Without 8 non-essential amino acids	47	σ18.5	31– 61
3	9	Without inositol	101		91–129
1	9	With kinetin	70		
3	9	With fat-soluble vitamins	74		51– 89
2	9	With ascorbic acid 80 mg. %	15		14– 15
2	9	With ascorbic acid 120 mg. %	23		14– 32
4	9	With glutathione 30 mg. % periodically replenished	30		11– 45
2	9	With cysteine 26 mg. %	0		—
4	9	Colchicine (2×10^{-8}M) added after 7 hr of culture	196 (prophases and metaphases)		156–297
14	24	No change	62	σ 26.0	20–103
5	24	without lactic acid	64		31– 90

and lactic acid were measured as described in a previous paper[2] and ascorbic acid was determined by titration with dichlorophenol-indophenol.

RESULTS

The effects on mitosis of changing the composition of the medium are summarized in Table 1. On the basis of these results, the KEI medium No. 1 has been modified by replacing alpha-ketoglutaric acid with glutamine, by adding TPN, and by omitting six nucleosides and copper sulfate. The composition of this medium (KEI No. 2) is given in Table 2. As indicated in Figure 1, it supports an approximately normal level of epithelial cells in division for nine hours of culture. This period can be extended to 18 hours, without adverse effect on mitotic activity, if the medium is exchanged for fresh solution after nine hours. The number of cells in division after 24 hours of culture without renewal of the medium declines to about 60 percent of in vivo. It was demonstrated with the use of colchicine that there was continued cell division during the last two hours of nine-hour cultures (table 1). The average ratio of metaphases in vitro to prophases and metaphases in vivo (1.96) was similar to that found by Constant.[8]

The amounts of glucose consumed and lactate produced by lenses cultured in this medium remained apparently normal and more or less proportional during seven days of culture (fig. 2). These lenses retained their initial ascorbic acid concentration over this period, but if this compound was excluded from the medium, its concentration declined to about one third after one week (table 3). The initial glutathione concentration of lenses was maintained for two days only.

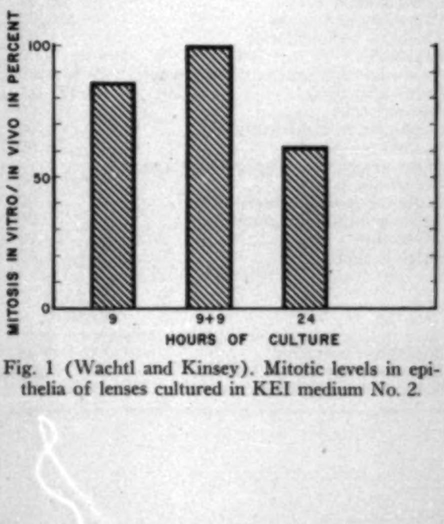

Fig. 1 (Wachtl and Kinsey). Mitotic levels in epithelia of lenses cultured in KEI medium No. 2.

TABLE 2
COMPOSITION OF KEI MEDIUM NO. 2

	Mg./Liter Medium
Sodium chloride	6,140.00
Sodium bicarbonate	2,980.00
Calcium chloride 2 H_2O	204.00
Magnesium sulfate 7 H_2O	124.00
Dibasic sodium phosphate 7 H_2O	188.00
Zinc chloride	0.04
Sodium citrate	7.00
Sodium carbonate	316.00
Ferrous sulfate 7 H_2O	2.50
Potassium iodide	0.30
Manganous sulfate H_2O	1.00
Monobasic potassium phosphate	8.00
Glucose	1,250.00
Creatine H_2O	33.00
Ammonium sulfate	2.00
Biotin	0.10
Pyridoxine hydrochloride	1.20
Calcium pantothenate	1.00
Niacin	1.00
Folic Acid	0.10
Cytochrome C	0.30
Inositol	0.10
Vitamin B_{12}	0.003
P-aminobenzoic acid	0.20
Choline chloride	100.00
L-leucine	57.00
L-isoleucine	57.00
L-phenylalanine	75.00
L-tyrosine	91.00
L-proline	67.00
L-hydroxyproline	77.00
L-Alanine	37.00
L-glutamic acid	105.00
L-threonine	38.00
L-aspartic acid	71.00
L-serine	53.00
L-arginine monohydrochloride	80.00
L-lysine monohydrochloride	80.00
Glycine	37.00
L-histidine monohydrochloride	80.00
L-tryptophan	70.00
L-valine	38.00
L-methionine	46.00
Potassium carbonate	326.00
Thiamine hydrochloride	1.00
Riboflavin	1.00
Adenosinetriphosphate disodium salt	4.00
Lactic acid 85%	(0.922 ml.)
Ascorbic acid	376.00
L-cysteine monohydrochloride	40.00
Sodium pyruvate	76.00
Alpha-tocopherol phosphate disodium salt	1.20
Coenzyme A	2.00
Diphosphopyridine nucleotide	2.00
Triphosphopyridine nucleotide	2.00
Glutamine	20.00
Succinic acid	4.00
	Per Liter Medium
Potassium penicillin	40,000 units
Dihydrostreptomycin sulfate	100 mg.
N-butyl-parahydroxybenzoate	0.2 mg.

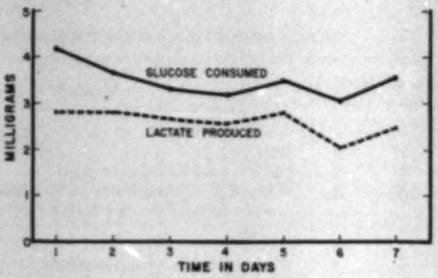

Fig. 2 (Wachtl and Kinsey). Glucose consumption and lactic acid production of lenses cultured for various periods.

High concentrations of ascorbic acid, glutathione, or cysteine had an inhibitory effect on mitosis. Attempts to simplify the medium showed that eight of the so-called nonessential amino acids could not be omitted. The presence of ascorbate, lactate, inositol, and fat-soluble vitamins (from Parker's medium No. 703[9]) was not necessary for nine-hour cultures (table 1).

Mitotic activity in general was independent of the redox potential of the medium within a range of +94 to +247 mv. and except in the cases of high levels of reducing compounds, it did not vary greatly with the substance used to alter the redox potential of the medium (table 4).

The influence of the temperature on cell division is shown in Figure 3. Though the

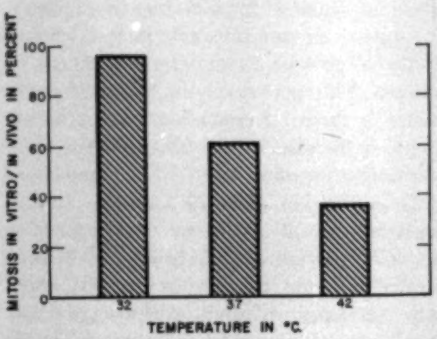

Fig. 3 (Wachtl and Kinsey). The effect of temperature on mitosis in epithelia of lenses cultured for 24 hours.

TABLE 3

ASCORBIC ACID AND GLUTATHIONE IN LENSES AFTER CULTURE, EXPRESSED AS PERCENTAGE OF CONTROL EYES

No. of Lenses	Days of Culture	Ascorbic Acid in Medium*	Ascorbic Acid in Vitro/in Vivo in Lens	Glutathione in Vitro/in Vivo in Lens
		Mg.%	%	%
10	1–2	40	112	108
5	3	40	122	74
2	7	40	109	53
5	3	None	54	88
2	7	None	29	23

* Present initially.

level of epithelial cells in division was normal after 24 hours of culture at 32°C., there were indications of a disorganization of the epithelial sheet. Mitosis was partially inhibited at 42°C.

Discussion

A marked improvement in the adequacy of the medium to support mitosis was achieved when alpha-ketoglutaric acid was replaced by glutamine and TPN was added simultaneously. Glutamine had been shown by Eagle[10] to be an essential constituent of tissue culture media and the beneficial effect observed could be due to it rather than TPN. The latter, however, was included in the medium in view of the possible importance of its presence for the phosphogluconate oxidation pathway which, as demonstrated by Kinoshita,[11] is relatively active in the lens. Though others found[12] that some nucleosides, such as adenosine, would inhibit mitosis, we could not detect a marked improvement in cell division when the nucleoside contituents of KEI No. 1 were omitted.

The reason for excluding copper sulfate was that the half life of ascorbic acid, when present in the medium at a concentration of 40 mg. percent, was doubled in the absence of copper ion (from two to four hours). Though the ascorbic acid is quite unstable in the medium, it does not necessarily disappear from the lens itself during culture. Bakker,[13] who used Ringer solution which had been equilibrated with intraperitoneal fluid as culture medium, reported that the ascorbic acid of the lens had vanished after the fifth day of culture. In the present study, the con-

TABLE 4

THE EFFECT OF VARYING THE REDOX POTENTIAL OF THE MEDIUM ON THE NUMBER OF EPITHELIAL CELLS UNDERGOING MITOSIS IN NINE-HOUR CULTURES

No. of Lenses	Redox Agent added to KEI Medium No. 2	Redox Potentials		Mitosis in Vitro/ in Vivo	Range
		Before	After		
		mv	*mv*	%	%
14	None	+247	+207	74	33–150
19	Ascorbic acid 25 mg.%	+139	+166	78	47–124
9	Ascorbic acid 40 mg.%	+101	+137	85	46–110
2	Ascorbic acid 80 mg.%	+ 78	+100	15	14– 15
2	Ascorbic acid 120 mg.%	+ 68	+ 91	23	14– 32
2	Glucoascorbic acid 40 mg.%	+ 96	+139	96	67–124
2	Isoascorbic acid 40 mg.%	+ 96	+121	94	81–107
2	Glutathione 20 mg.%	+122	+211	83	82– 85
2	Glutathione 40 mg.%	+ 94	+196	72	67 –76
4	Glutathione periodically replenished 30 mg.%	+ 78	+172	30	11– 45
2	Cysteine 26 mg.%	+102	+142	0	—

centration of ascorbic acid remained at a normal level after seven days of culture.

At present, it is not known why mitosis declines in the course of 24 hours of culture without exchange of the medium. Occasional checks were made and it was observed that cell division in the epithelium stopped between the second and the third day of culture. The fact that the glucose consumption and lactate production of lenses were found not to vary greatly in the course of seven days indicates that though the lens stopped growing, it was still surviving for longer periods of time.

Summary

A medium (KEI No. 2) is described which was shown to support an approximately normal level of lens epithelial cells in division during nine hours of culture. The culture period can be extended to 18 hours without adverse effect on mitosis if the medium is exchanged for fresh solution after nine hours. The number of dividing cells after 24 hours of culture without renewal of the medium declines to about 60 percent of that in vivo.

The glucose consumption and lactate production of lenses cultured in this medium remain at normal levels for at least seven days. The concentrations of ascorbic acid and glutathione in cultured lenses were maintained for seven and two days, respectively.

Eight of the so-called nonessential amino acids were shown to be necessary for the maintenance of mitosis, but the presence of ascorbic acid, inositol, lactic acid, and fat-soluble vitamins is not required, at least for periods up to nine hours.

Mitotic activity, in general, was independent of the redox potential of the medium within a range of +94 to +247 mv. The effect of the temperature of the medium on mitosis was also determined.

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Technical assistance was given by Miss Jean Gorman.

References

1. Merriam, F. C., and Kinsey, V. E.: Studies on the crystalline lens: I. Technic for in vitro culture of crystalline lenses and observations on metabolism of the lens. Arch. Ophth., **43**:979, 1950.
2. Kinsey, V. E., Wachtl, C., Constant, M. A., and Camacho, E.: Studies on the crystalline lens: VI. Mitotic activity in the epithelia of lenses cultured in various media. Am. J. Ophth., **40**:216 (Nov. Pt. II) 1955.
3. Wachtl, C., and Kinsey, V. E.: Studies on the crystalline lens: VII. Utilization of several carbohydrates and their effects on mitosis of lens epithelium. Am. J. Ophth., **44**:318 (Nov. Pt. II) 1957.
4. Kinoshita, J. H., and Wachtl, C.: A study of the C^{14}-glucose metabolism of the rabbit lens. J. Biol. Chem., **233**:5, 1958.
5. Howard, A.: Whole mounts of rabbit lens epithelium for cytological study. Stain Technology, **27**:313, 1952.
6. von Sallmann, Ludvig: Experimental studies on early lens changes after roentgen irradiation: III. Effect of x-radiation on mitotic activity and nuclear fragmentation of lens epithelium in normal and cysteine-treated rabbits. Arch. Ophth., **47**:305, 1952.
7. Schwartz, B., anl Leinfelder, P. J.: Ascorbic-dehydroascorbic acid as an oxidation-reduction system in the maintenance of the metabolism of lens cultured in vitro: I. Weight and cation equilibrium. Am. J. Ophth., **40**:224 (Nov. Pt. II) 1955.
8. Constant, M. A.: In vitro lens studies: I. Technique and preliminary results of maintenance of lens culture for 24 hours. Am. J. Ophth., **45**:372, 1958.
9. Healy, George M., Fisher, Dorothy C., Parker, Raymond C.: Nutrition of animal cells in tissue culture: IX. Synthetic Medium No. 703. Can. J. Biochem. and Physiol., **32**:327, 1954.
10. Eagle, Harry: Nutrition needs of mammalian cells in tissue culture. Science, **122**:501, 1955.
11. Kinoshita, Jin H.: Carbohydrate metabolism of lens. Arch. Ophth., **54**:360, 1955.
12. Hughes, Arthur: Effects of purines and related substances upon cells in chick tissue cultures. Exper. Cell Res., **3**:108, 1952.
13. Bakker, A.: Ueber die Bedeutung der Askorbinsäure (Vitamin C) für den Stoffwechsel der Linse. Arch. f. Ophth., **136**:166, 1936.

Discussion

Dr. Marguerite A. Constant (St. Louis): The investigators have made several fundamental contributions to the maintenance of the lens in vitro. First, they have improved the media so that mitosis is successfully maintained now, not for nine hours as previously, but for 18 and possibly 24 hours and longer.

This criterion would seem inconsequential in terms of quantity of epithelium in relation to the quantity of lens or quantity of cells in mitosis in relationship to the total number of cells in the epithelium. It does, however, add one important support to the fact that the lens may be maintained in vitro as it existed in vivo.

The importance of this in vitro criterion in relationship to pathologic changes or enzymatic activity in the maintenance of the lens can be only inferred at the present time by analogy of the work by von Sallmann in vivo. von Sallmann's work strongly implicates mitotic activity and cataract formation in animals.

Secondly, the authors have achieved simplification of the media. This is very important from a practical standpoint and from the point of interpreting interrelationships of ingredients of the media and lens activity. I hope the authors continue their work along this line to a successful media of about 10 ingredients. I might be convinced then to switch from the commercially available TC-199 to this simple media.

I should like to add that TC-199, which maintains the lens successfully for about 12 hours, does not contain ascorbic acid to any significant amount; but if one adds ascorbic acid it not only inhibits mitosis but also produces marked degenerative changes in the epithelial cells. The level of ascorbic acid added was only 20 mg. percent. Thus, there is a marked difference between in vitro studies and the in vivo lens in so far as ascorbic acid is concerned. The interrelationship of ratios of substances has been well shown for the nutrition of the whole animal, and is probably very true for the nutrition of the lens in vitro.

Thirdly, the investigators have implied the importance of the potential of the media to the maintenance of mitotic activity. Several investigators using tissue culture have shown that when the potential falls to approximately +50 mv., mitosis and proliferation cease.

I should like to ask Dr. Wachtl whether he found any correlation in his longer culture studies between the media potential falling to less than 95 mv. and the failure of mitosis.

This is a practical correlation for, as he well knows, mitotic counts are very tedious. It also would have theoretic importance because, if the lens differs in this respect from general tissue culture studies, or if it is rather similar, one can call upon the extensive work done in other systems in attempting to get at the lens problem.

Dr. Carl Wachtl (closing): We would like, and I am sure I speak also for Dr. Kinsey, to reduce the number of constituents of our medium to 10 or less. Work toward simplification of it will be continued. I would hesitate, however, at present to make any predictions.

The observation of Dr. Constant that ascorbic acid when added to TC-199 inhibits mitosis is quite interesting. It shows that the effect of a particular component may vary depending on its proportion to or interaction with other constituents and perhaps also on the conditions of culture. Nature, as Dr. Potts remarked this morning, is wonderful. We deal with very delicately balanced systems and thus, what may be true under certain conditions, may not be true under others.

We did not measure potentials over culture periods longer than nine hours. When ascorbic acid was present in the medium in a concentration of 120 mg. percent, the initial redox potential was +68 mv. and mitosis was depressed, while at +96 mv. it was approximately normal. At present we are not able to state whether this partial inhibition of mitosis was due to the low potential or to the high concentration of the reducing agent. Unless there is a critical potential, below which cell division decreases sharply, there is no evidence that the reduced mitotic activity was due to the low potential only. Dr. Constant's recommendation to measure the potentials over longer periods of culture is a good one and experiments of that kind may provide an answer to this question.

I would like to thank Dr. Constant for her discussion and stimulating suggestions.

A TISSUE CULTURE TECHNIQUE FOR GROWING CORNEAL EPITHELIAL, STROMAL, AND ENDOTHELIAL TISSUES SEPARATELY*

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Introduction

Several methods have been recently employed for determining the effects on and viability of corneal tissue that has been preserved and stored. These procedures have included the following:

a. Electrophoretic studies of the proteins of the cornea.[1]

b. Grafting of corneas onto the chorioallantoic membrane of 10-day-old chick embryonated eggs.[2]

c. Tissue culture studies.[3]

d. Regular lamellar and penetrating grafting operations.[4]

The clinical results have been judged as a success or failure without determining the cause. In the above mentioned procedures little has been said about which tissue (s), that is, epithelium, stroma, or endothelium, actually had survived the preservation and storage treatment. If this knowledge were obtained and correlated with a clinical evaluation of similarly treated corneas, then an assessment of the importance and role of each tissue in determining the success of corneal grafts would be possible.

Matsui[5] in 1929 reported on the successful removal and cultivation in vitro of Descemet's membrane and endothelial cells of rabbit corneas. As far as we can determine this is the only report of separating the cornea into different layers and culturing them individually.

The purpose of this paper is to present a procedure for separating the cornea into epithelial, stromal, and endothelial layers, and culturing them in vitro. A separate paper[6] has described the results obtained with preserved and stored corneas.

Materials and Methods

Adult rabbits were sacrificed by inoculation of sodium pentobarbital into the marginal ear vein, and the corneas were immediately removed under aseptic conditions. This is usually within the limbus so no scleral or conjunctival tissue remains. The excised corneas were transferred to sterile beakers containing Hanks' balanced salt solution (BSS)[7] without sodium bicarbonate, and containing penicillin and streptomycin in concentrations of 100 units and 100 µg. per ml., respectively. After soaking for approximately five minutes, a cornea was transferred to a small petri dish containing fresh BSS without sodium bicarbonate and without antibiotics with the epithelial layer on the bottom. This petri dish was placed under a dissecting microscope, the bulk of the fluid removed, and the tissue separated into three parts. Two fine pointed forceps were em-

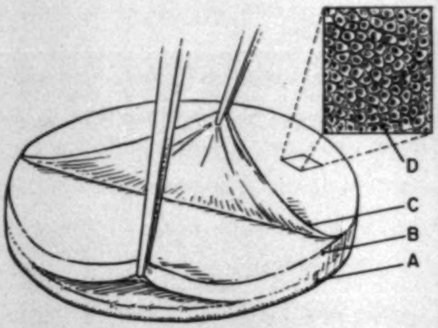

Fig. 1 (Stocker, et al.). Composite drawing to illustrate the technique of separating the cornea into three layers. (A) Epithelial layer. (B) Stromal layer. (C) Descemet's membrane plus endothelial cells. (D) Surface view of endothelial cells.

* From the Duke University School of Medicine Plastic Surgery Research Laboratory and Division of Ophthalmology. This work was aided by U. S. Public Health Grant B-1161. This work was also aided by Playtex Park Research Institute Grant No. TR65.

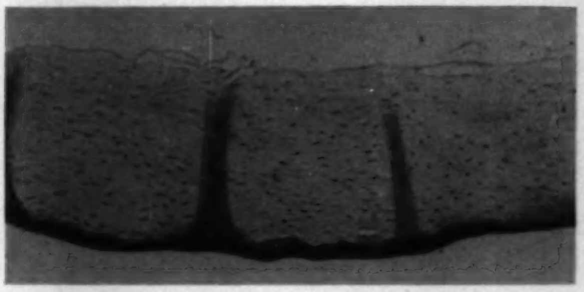

Fig. 2 (Stocker, et al.). The epithelial layer (dark staining) has attached to it a portion of the stromal layer (light staining). (X82).

ployed, one to anchor the tissue and the second to gently peel Descemet's membrane from the cornea.

This step is rather delicate in that the endothelial cells are easily removed by excessive handling and scraping. If the cornea remained in the solution more than 15 to 20 minutes, the endothelial cells began floating off into the medium due to the absorption of fluid. The strips of Descemet's membrane had the endothelial cells attached and were transferred directly to 11 by 22-mm. cover slips which had been previously coated with either chicken plasma or rabbit plasma. After complete removal of the Descemet's membrane-endothelial cell layer, the remaining tissue was again separated into two parts by using two fine forceps as before. The top layer was the stromal tissue while the bottom one was the epithelial layer.

Figure 1 shows a composite drawing of these two steps. Figures 2, 3, and 4 show the appearance of the three layers after separation. The last two layers were cut into small pieces (3 to 4 mm.^{2}) and were also transferred to cover slips. The plasma was then clotted by addition of 20-percent chick embryo extract to the cover slip preparations. The cover slips were transferred to Porter flasks and 1.0 ml. of fluid medium added. Silicone-treated stoppers were inserted and the flasks placed in the 37°C. incubator. The cultures were observed three times a week for a three-week period. The fluid medium was changed whenever the medium became acid as shown by the phenol red indicator. Four fluid media have been employed:

a. Rabbit serum—40 percent, Hanks' balanced salt solution—60 percent.

b. Rabbit serum—30 percent, Mixture 199-70 percent.

c. Pooled human serum 40 percent, Hanks' BSS-60 percent.

d., Pooled human serum 30 percent, Mixture 199-70 percent.

Penicillin and streptomycin in final concentration of 100 units and 100 micrograms were added to all media. Two flasks were set up for each layer for a total of six flasks per cornea.

A second method that has been employed for the romoval of the epithelial and/or endothelial cells was the mechanical scraping

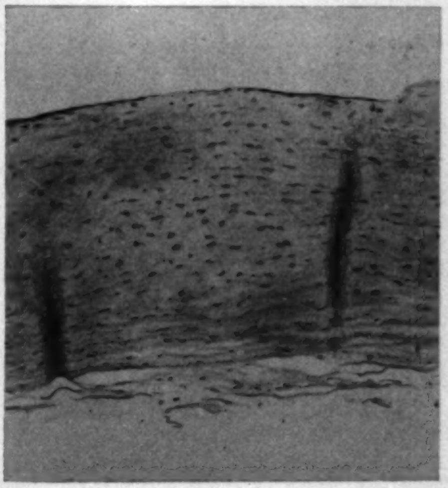

Fig. 3 (Stocker, et al.). This illustration shows the stromal tissue which has been separated from the other layers. (X82.)

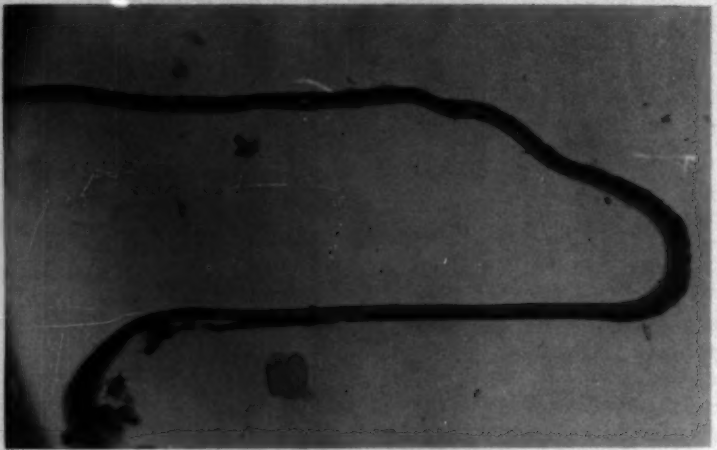

Fig. 4 (Stocker, et al.). The single layer of darker staining endothelial cells is shown attached to Descemet's membrane. ×350.

of these layers into two drops of chick plasma, transferring this fluid-cell mixture to a cover slip, clotting it with chick embryo extract, and proceeding as before. However, not all of the epithelial or endothelial cells were completely removed each time by this method, and growth of these remaining cells occurred in the stroma cultures, yielding a mixed growth.

Results and Discussion

During the development of this technique, the first 24 cultures produced no endothelial growth. This was chiefly due to initial technical difficulties, that is, no cells remained after separation or the cells became dehydrated. Since then, we have been using a dissecting microscope which has greatly facilitated the process and have had practically

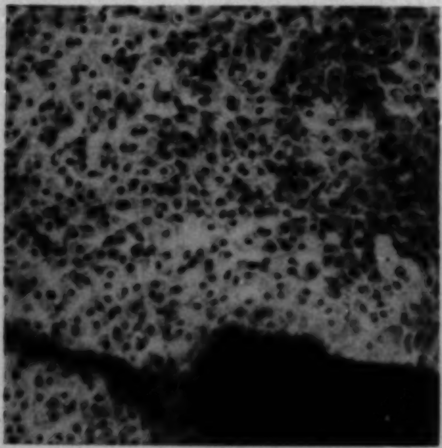

Fig. 5 (Stocker, et al.). The darker staining original explant is shown with an outgrowth of rabbit epithelial cells, seven days in culture. (X140.)

Fig. 6 (Stocker, et al.). A 10-day rabbit stroma culture is shown with the darker area being the original explant. X82.)

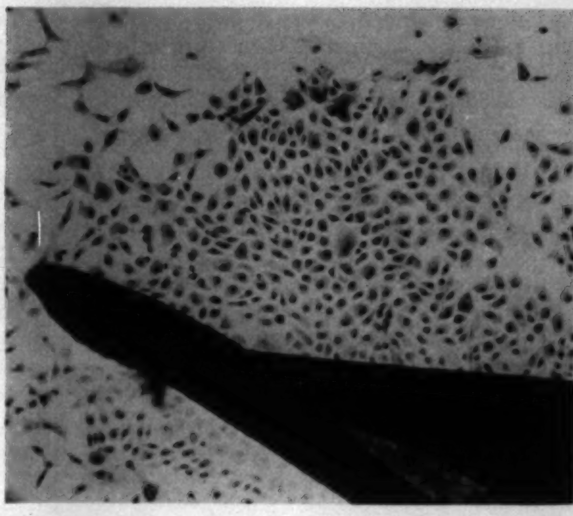

Fig. 7 (Stocker, et al.). The outgrowth of endothelial cells is shown in this five-day rabbit culture. The darker area is the original explant composed of Descemet's membrane and endothelial cells. (X140.)

100-percent growth in every culture for the past year and a half (39 out of 40 cultures have grown). The epithelial and stromal culture have also been grown successfully in all cases (over 60 cultures of each).

The epithelial and endothelial cells commence migration and proliferation in one or two days and have a good growth in three to five days. In contrast, the stromal cells have a lag period of three to five days and then begin migration and proliferation with seven to 10 days required for a good outgrowth to occur. Figure 5 shows the outgrowth from a seven-day-old culture of the epithelium; Figure 6 is of a 10-day culture of the stromal layer; Figure 7 shows endothelial outgrowth in seven days; and Figure 8 is of an endothelial cell in mitosis.

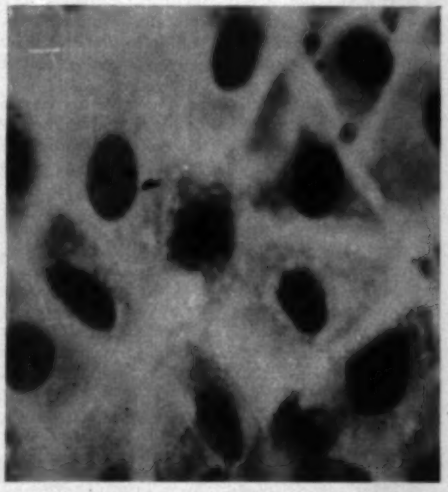

Fig. 8 (Stocker, et al.). At this magnification of a rabbit endothelial culture, mitotic figures are easily seen. (X700.)

At first, an autologous medium as far as practical was employed, that is, rabbit plasma and rabbit sera from the same animal were used. Homologous and heterologous sera and plasma were employed later and found to be equally successful in promoting migration and proliferation of cells from all three tissues.

The epithelial "layer" as shown in Figure 2 is composed of both epithelial and stromal tissue. In culture, only epithelial cells migrate and proliferate. The point of cleavage is of utmost importance in that if the cleavage is closer to the Descemet's membrane side, both epithelial and fibroblast growth results in the epithelial culture, whereas if the cleavage is close to the epithelial side, only a epithelium type of growth is observed.

Summary

A tissue culture technique for separating

and growing the three tissues of the cornea, that is, epithelium, stroma, and endothelium, in vitro, has been developed. Utilization of this method in studying preserved corneas has permitted evaluation of the role of each tissue in grafting operations.[6]

Duke University
School of Medicine.

References

1. Fielding, I., Basu, P. K., and Ormsby, H. L.: Electrophoretic studies on stored corneas. Am. J. Ophth., **44**:385, 1957.
2. Basu, P. K., Miller, I., and Ormsby, H. L.: Viability of stored cornea of the rabbit on the chorioallantoic membrane of the chick. Am. J. Ophth., **44**:209, 1957.
3. Draheim, J., McPherson, S. D., Evans, V. J., and Earle, W. R.: Further studies on the viability of frozen corneas as determined in tissue culture. Am. J. Ophth., **44**:182, 1957.
4. King, J.: Keratoplasty: Experimental studies with corneas preserved by dehydration. Am. J. Ophth., **43**:352, 1957.
5. Matsui, J. Ueber die in vitro Kultur des Endothels der Membrana Descemetii. Arch. f. Exper. Zellforschung, **8**:533, 1929.
6. Stocker, F. W., Eiring, A., Georgiade, R., and Georgiade, N.: Evaluation of viability of donor tissue prior to corneal grafting. Tr. Am. Ophth. Soc., 1958. in press.
7. An Introduction to Cell and Tissue Culture. The staff of the tissue culture course, 1949 to 1953 in Cooperstown, N.Y., Minneapolis, Burgess, 1955, p. 7.

Discussion

Dr. Carl Wachtl (Detroit): The authors have presented a useful technique for the separation of corneal tissue into epithelium, stroma, and endothelium, and have shown how each layer can be maintained separately in tissue culture.

Kirby, in 1927, cultured corneas from chick embryos and observed an outgrowth which was composed of epithelial, fibroblast-like, and plate-like cells. Attempts to isolate the epithelium, however, were not successful.

From the slides presented by Dr. Georgiade it can be seen that each layer produces an outgrowth fairly typical of the respective cell types. The cells migrate evenly and the occurrence of mitosis, for instance in the endothelium, demonstrates that these cells are proliferating. Some caution, of course, has to be exercised when observations are interpreted and conclusions drawn with respect to the viability of the cornea as a whole and its subsequent use for corneal grafts.

That separated layers of a tissue which are composed of different types of cells migrate and proliferate under certain conditions, is a sign of viability but not necessarily a proof that the tissue as a whole will be functional. Slight changes may have occurred which could show an effect if these layers would be combined.

Instances are known where one type of cell requires the presence of another type in order to carry out some of its functions. Thus, for instance, the presence of fibroblasts is necessary for the differentiation of certain epithelial cells.

It will be inteersting to hear of the experience the authors had with preserved and stored corneas, and to learn of correlations of their findings with clinical observations. There are perhaps also other uses for this tissue culture technique. It could be employed, for instance, in studies of the metabolism and the specific biochemical requirements of endothelium and stroma, now that these can be separated into growing organized layers. The effects which metals and other substances injurious to the cornea have on the different component tissues could be examined with respect to wound healing.

These are just some of the possible applications of this technique which I would like to mention. There are probably still others, and, I believe, the authors should be congratulated on their successful endeavors.

Dr. N. Georgiade (closing): I wish to thank Dr. Wachtl for his very kind comments. I would like to mention that in a previous paper, presented a few weeks ago at the American Ophthalmologic Society, we did bring up the point of preservation of the corneas, and we were able to preserve them for 12 weeks, versus the usual preservation time of 24 to 48 hours.

We have been able to preserve these corneas in a Hanks' balanced salt solution with 20 percent glycerol added, and we were able to regrow all the layers separately, as outlined in our paper this afternoon.

This brings up the problem potentially of being able to preserve corneas for an indefinite period of time in a viable state. In our laboratories we have been able to preserve skin, for example, for over 1,000 days in a viable state. We have been able to obtain positive tissue cultures of the skin, and we have been able to transfer the skin back to the laboratory animal (in this particular case a dog) and have it grow. Sections confirming this have been done as well.

So, the possibility in corneal grafting with the use of preserved media, I think, is very interesting and intriguing, and perhaps by changing our solutions from time to time as we have had to do in the past to get to the 12-week period we have now, we can prolong it for a considerably greater period of time.

STUDIES ON THE CILIARY EPITHELIUM AND THE ZONULE*

I. Electron microscope observations on changes induced by alteration of normal aqueous humor formation in the rabbit

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New York

Although the structure of the zonule of Zinn and of the ciliary epithelium has been the subject of many investigations,[1] our knowledge of the fine structure has formerly been limited by the resolving power of the light microscope. Using the techniques of electron microscopy, two aspects of the zonular fibers and of the ciliary epithelium were studied: (1) their structural role in accommodation, and (2) their relationship to aqueous humor dynamics.

The two cell layers of the ciliary epithelium are bounded by the posterior chamber with the zonule of Zinn on one side and connective tissue that makes up the main body of the ciliary process on the other. With the aid of the higher resolving power of the electron microscope, the method by which zonular fibers are inserted onto the ciliary epithelium will be considered, as well as the relationship of the connective tissue stroma to the epithelial cells.

The ciliary epithelium is known to be involved in the formation of aqueous humor.[2] Electron microscope observations on the ciliary epithelial cells have shown elaborate infoldings and interdigitations of the cell membrane, indicative of secretory activity.[3-6] Observations on changes induced in these structures by Diamox, bacterial endotoxin, and blockage of the drainage angle with mineral oil have been made and are the subject of this communication.

Materials and Methods

Young New Zealand albino rabbits weighing approximately 3.0 kg. were used. The animals were lightly anesthetized with nembutol. In some cases the fixative was injected into the posterior chamber with a 27-gauge needle and the aqueous was allowed to escape through a keratome incision in the cornea. Immediately after this injection the eye was enucleated, the cornea removed, and more fixative pipetted onto the ciliary processes, while the iris was lifted by the operator. This procedure was done under low magnification and it could be seen that the processes were fixed by the injected osmic acid mixture. Groups of two or three processes from the corona ciliaris were removed and placed in a large volume of the osmic acid solution.

A variation of this method was also used in which the eye was rapidly enucleated, opened, and the processes fixed by pipetting onto them a quantity of the fixative fluid. In two instances this was done while the eye was in situ. The tissue was always cut with sharp razor blade knives. Good fixation was found to follow all of these methods.

The fixative used for electron microscopy was one percent osmium tetroxide buffered with veronal-acetate at pH 7.8. After fixation for 15 to 20 minutes, the specimens were washed and dehydrated in alcohol, infiltrated with n-butyl methacrylate, prepared according to Moore and Grimley.[7] The tissue was embedded in methacrylate by polymerization at 60°C. Two-percent luperco was used as a catalyst. In some of the preparations (from which figs. 9 and 10 were taken) 0.075-percent uranyl nitrate was added to the luperco-methacrylate solution.* The embedded speci-

* From the Departments of Anatomy and Ophthalmology College of Physicians and Surgeons, Columbia University. This investigation was supported in part by research grants B 1202 (C1) and B 492 (C4) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

* R. Ward, personal communication.

mens were sectioned with a Porter-Blum microtome. The sections were mounted on grids, previously coated with formvar to provide a supporting film. The sections were examined in an RCA microscope, model EMU-3C.

Results

Observations on normal structures

Figure 1 shows a section through the two cell layers of the ciliary epithelium. The cell layer facing the posterior chamber (PC) is more prominent and larger than the pigment cell layer, which rests upon the basement membrane (BM) of the connective tissue stroma. In this study pigment is not present in these cells, since albino rabbits were used.

Epithelial cells facing the posterior chamber are characterized by two types of cell membrane elaborations: (1) prominent infoldings of the cell membrane on the apical surface (A, in figs. 1, 2, and 4), and (2) very elaborate interdigitations of the cell membranes between these cells (B, in figs. 1, 2, 3, and 4). This elaborate interdigitation of adjacent cell margins can appear in various degrees of complexity (figs. 2 and 3), and extends two thirds of the distance between the apical portion of contact and the basal portion of the cell.

Elements of the endoplasmic reticulum with its associated granules[8] can be found ("ER" in figs. 2, 3, and 5) in the cytoplasm of the ciliary epithelial cells. Mitochondria can be found scattered throughout the cytoplasm (figs. 1 and 5). Agranular, flattened vesicles of the Golgi complex[9] are found in the area around the basal portion of the nucleus (fig. 5). Light microscope preparations of the ciliary processes were found to contain rather little Golgi material. In Figure 5 an oblique section through the nuclear envelope shows nuclear pores (P) to advantage.[10]

Still another specialization of the cell membrane can be found in the basal portion of the epithelial cells which is in contact with the pigment cells. Regional thickenings of the cell margins, or desmosomes, are seen (fig. 11). The regional thickenings of each cell are adjacent to those of the neighboring cell so that they correspond and form the structures known as desmosomes or "adhesion plates." These structures are found in epidermis.[11,12] Small fingerlike processes of pigment cell cytoplasm project into the basal portion of the epithelial cell (fig. 12). Similar intercellular fingerlike processes have also been seen joining liver cells.[13]

The apical surface of the epithelial cells facing the posterior chamber is lined with the internal limiting membrane (*Membrana limitans interna ciliaris*). The internal limiting membrane (150 to 300 Å thick) is in close apposition to the contours of the cell surface (fig. 4), but does not line the characteristic infoldings and interdigitations of these cells. Zonular fibrils enter the internal limiting membrane but do not penetrate any closer to the epithelial cells (fig. 6). Thus, the internal limiting membrane represents the point of most intimate relationship of the zonular fibers with the ciliary epithelium. In sections where the internal limiting membrane is cut obliquely, it is seen to be fibrillar in nature and made up of zonular material (figs. 7 and 8). This membrane, therefore, appears to be a condensation of zonular fibrils on the surface of the cells.

Sections through clusters of zonular material near the surface of the ciliary epithelial cells show clearly that the zonular fiber is made up of many small unit fibrils. In our preparations, the fibrils are of indefinite length and have a width of about 80 to 120 Å (fig. 9). In higher magnifications, a periodicity of 80 to 110 Å is evident in the unit fibril (fig. 10) which appears to be unbranched. There are indications that one or two subperiods may be present.

The basal margin of the pigment cells which rests on a basement membrane is very irregular (figs. 13 and 14). The fine fibrillar material of the basement membrane follows the irregular contour of the cell margin.

Fig. 1 (Pappas and Smelser). Electron micrograph of a section of ciliary epithelium from an albino rabbit. The epithelial cell layer facing the posterior chamber (PC) has many infoldings of cell membrane (A). Interdigitation of the margin between two cells can be seen (B). The pigment cell layer can be seen resting on a basement membrane (BM). In the cell, nuclei (N), mitochondria (M), and elements of the endoplasmic reticulum (ER) can be recognized. (×9,500.)

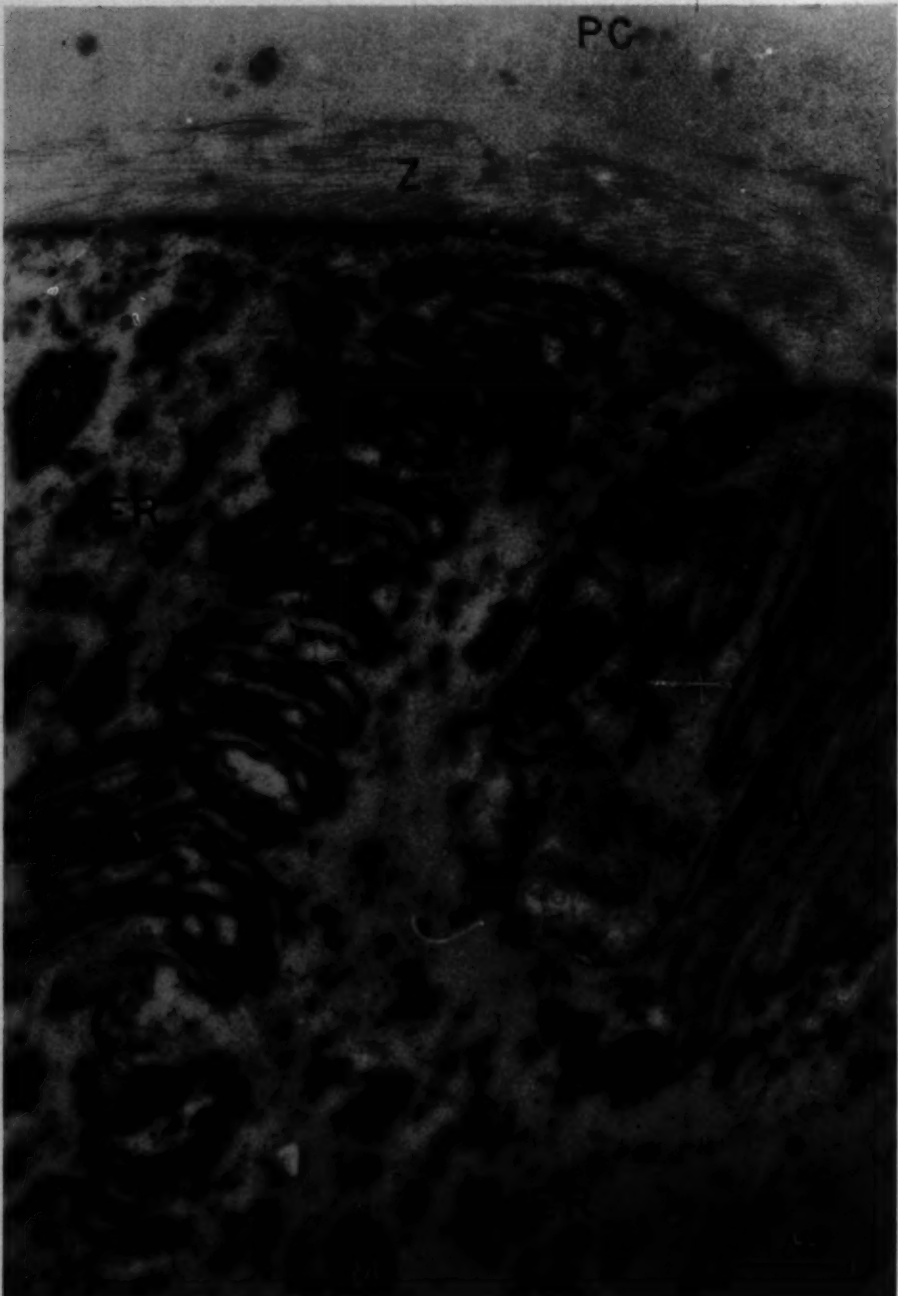


Fig. 2 (Pappas and Smelser). Electron micrograph of a section of portions of two adjacent ciliary epithelial cells showing complex interdigitations (B) of their boundaries. Infoldings (A) of the surface facing the posterior chamber (PC) also are shown. Zonular fibers (Z) can be seen in close approximation to the surface of these cells. Elements of the endoplasmic reticulum (ER) as well as mitochondria (M) are found in the cytoplasm. (×21,000.)

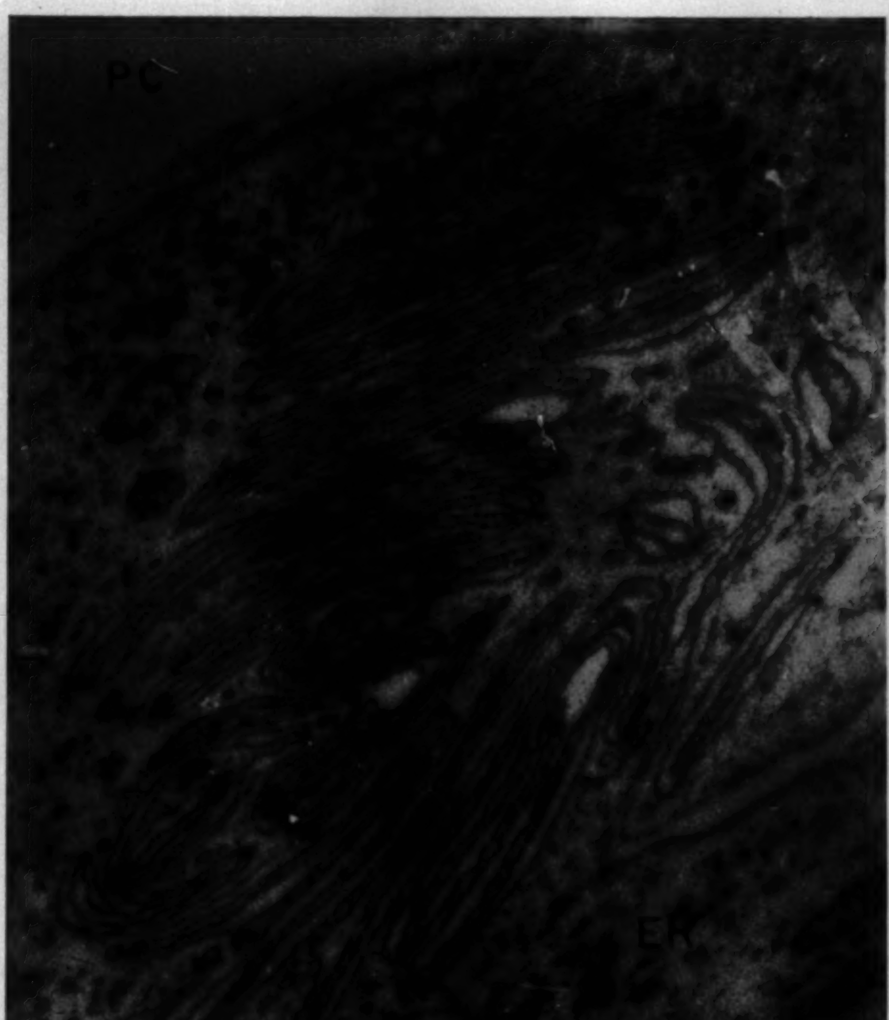


Fig. 3 (Pappas and Smelser). Electron micrograph of a section showing a very elaborate pattern of interdigitation of adjacent cell margins of two ciliary epithelial cells. Their free surface faces the posterior chamber (PC) and shows zonular fibers (Z) in close approximation. Mitochondria (M) and elements of the endoplasmic reticulum (ER) are found scattered in the cytoplasm. (×23,000.)

Fig. 4 (Pappas and Smelser). Electron micrograph of a section through two adjacent ciliary epithelial cells. The area from which the section is cut lies below the free surface of the cells facing the posterior chamber. A deep crevice formed by the irregular surface of the cells and lined with the internal limiting membrane (I) can be seen. The internal limiting membrane does not line the cell membrane infoldings (A) or the interdigitations (B). Mitochondria (M) are swollen due to Diamox injection. N = nucleus. (×19,500.)

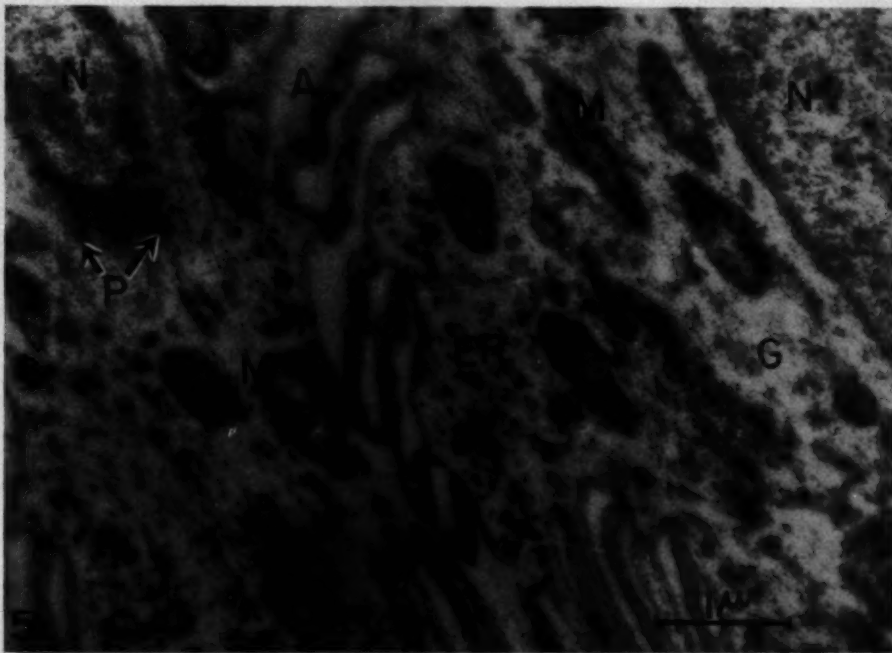
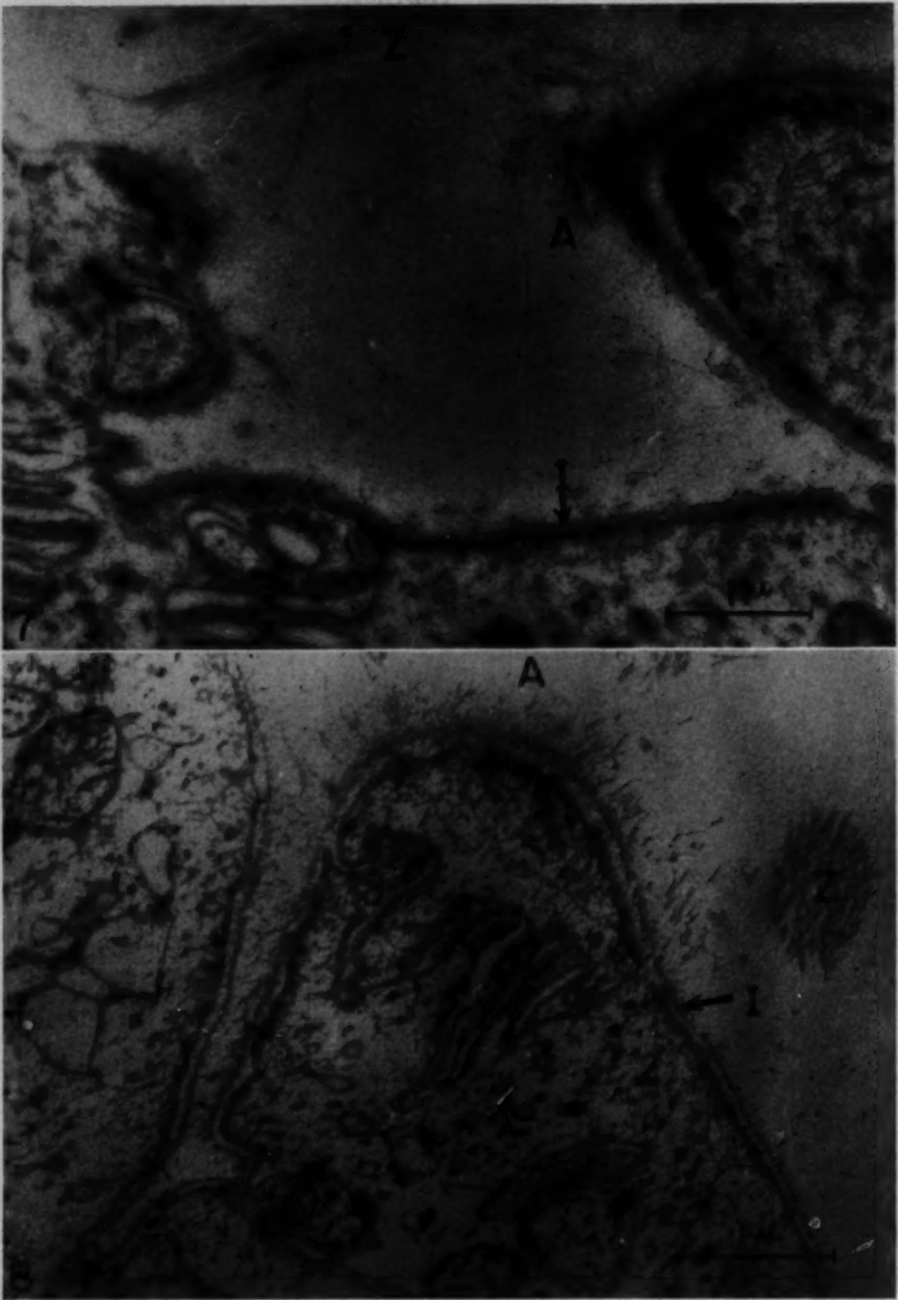


Fig. 5 (Pappas and Smelser). Electron micrograph showing a portion of two ciliary epithelial cells. The interdigitated margin (A) between these cells is evident. Elements of the endoplasmic reticulum (ER), and the Golgi complex (G), as well as mitochondria (M), are seen. The nuclear envelope of the nucleus (N) in the upper left is cut tangentially so that pores (P) can be seen. (×20,300.)

Fig. 6 (Pappas and Smelser). Zonular fibrils (Z), about 100 to 120 Å wide come up to the surface of the ciliary epithelial cell, forming the internal limiting membrane (I). (×21,200.)



Figs. 7 and 8 (Pappas and Smelser). Electron micrographs of sections of the surface of the ciliary epithelial cells facing the posterior chamber. This surface is covered with the internal limiting membrane (I). Oblique profiles (A) of the internal limiting membrane show that this layer is made up of zonular fibrils (Z). (Figure 7, ×22,000; Figure 8, ×24,000.)

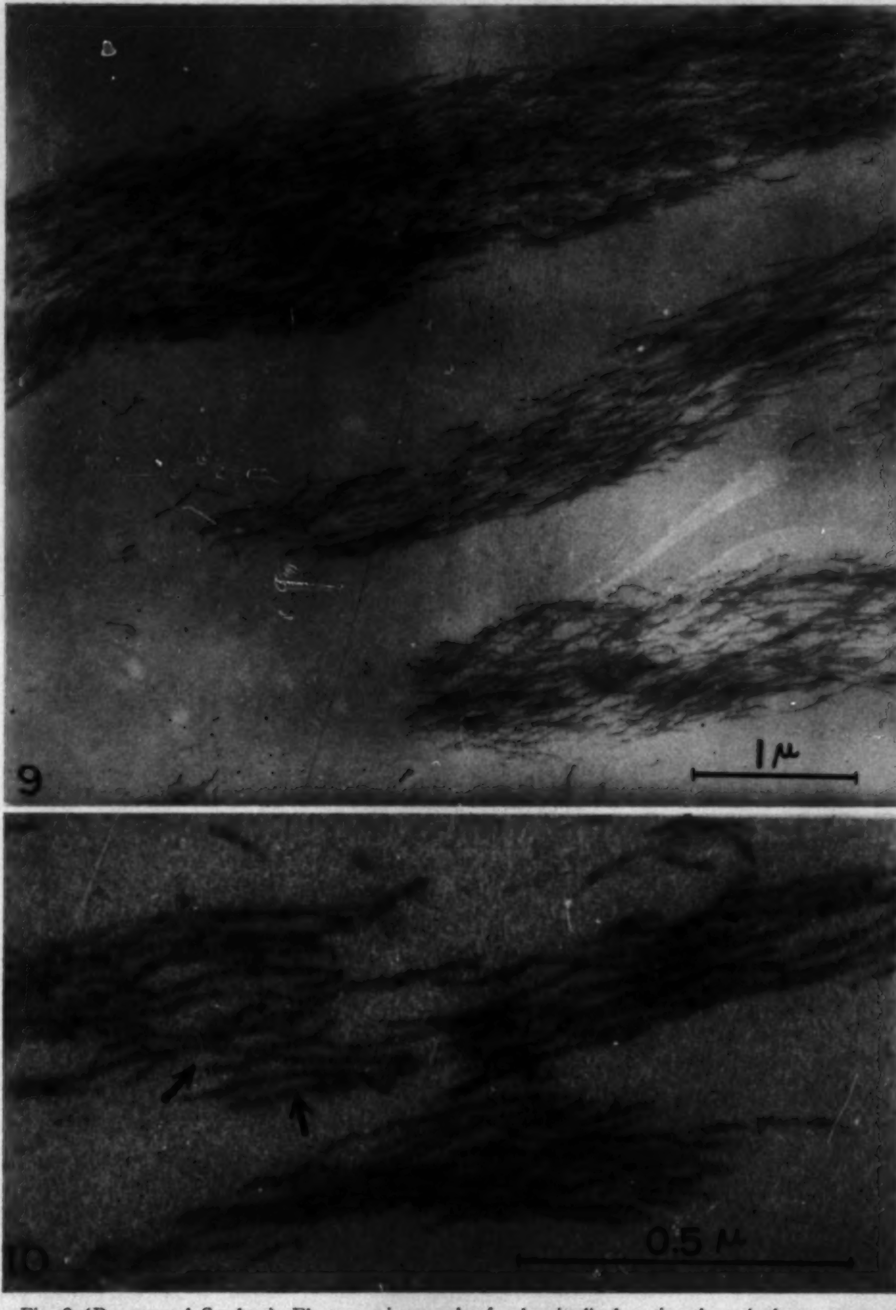
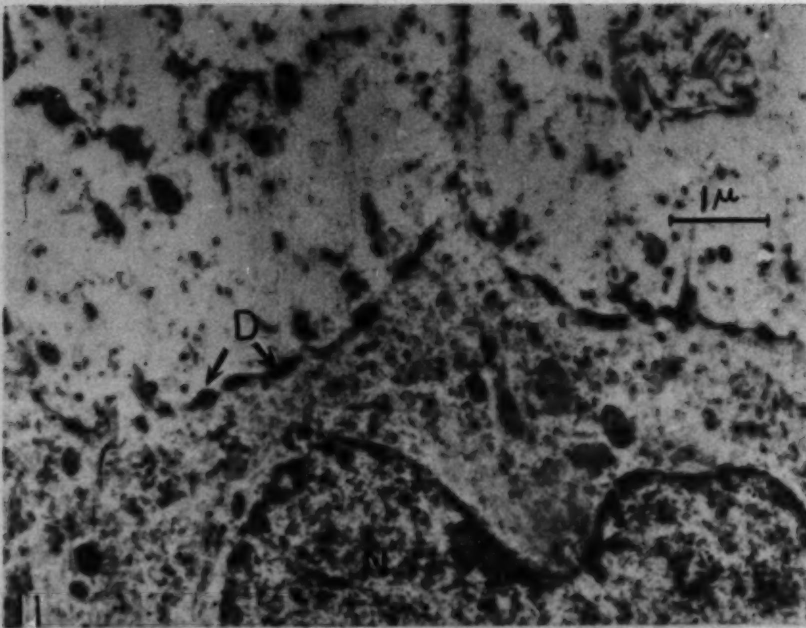


Fig. 9 (Pappas and Smelser). Electron micrograph of a longitudinal section through three groups of zonular fibrils. The individual fibrils are about 80 to 120 Å wide. (×25,200.)

Fig. 10 (Pappas and Smelser). Higher magnification of a group of zonular fibrils. A periodicity of 80 to 110 Å is apparent at arrows. (×103,600.)



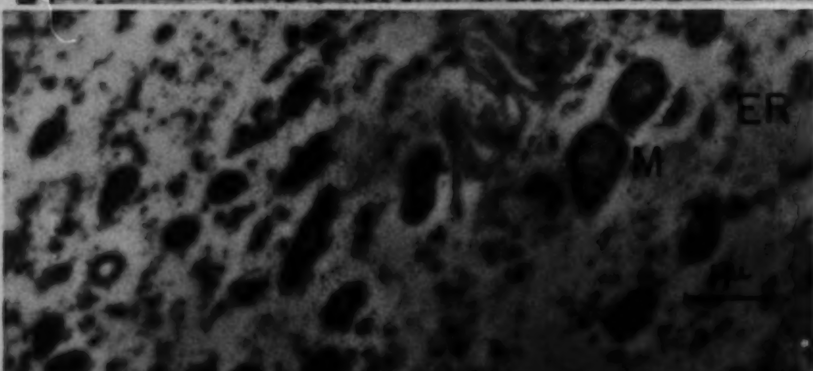
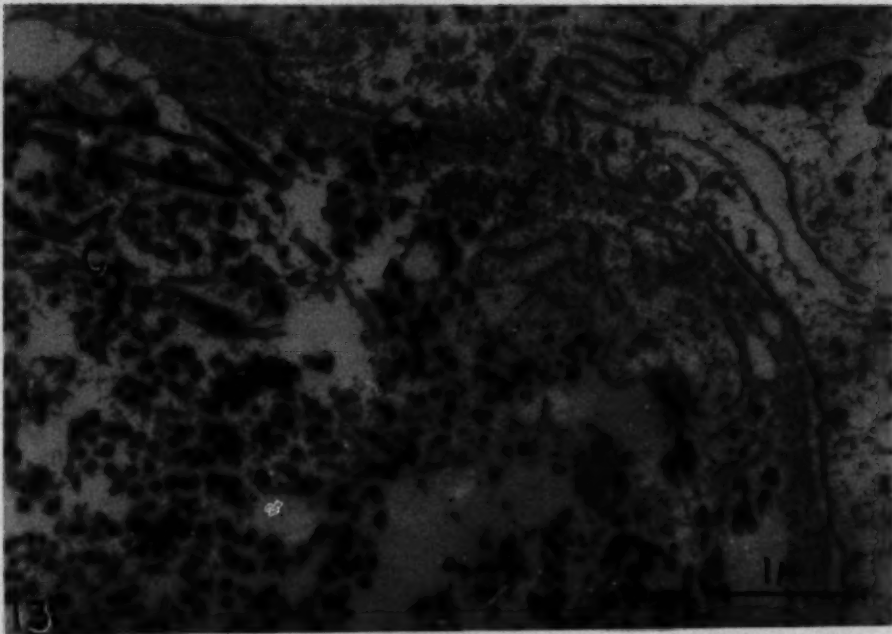
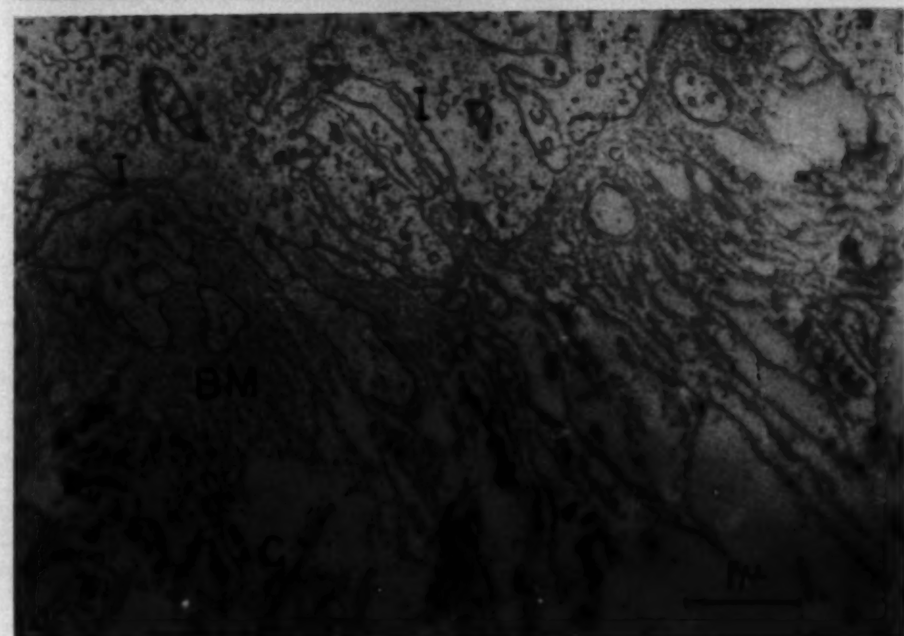


Fig. 11 (Pappas and Smelser). Electron micrograph of a section showing the junction between two epithelial cells and a pigment cell. A portion of the nucleus (N) of the pigment cell is seen. Corresponding thickenings of the cell membranes (at arrows) are desmosomes or "adhesion plates." (×15,300.)

Fig. 12 (Pappas and Smelser). Section also showing junction between two epithelial cells and a pigment cell. Finger-like projections of pigment cell cytoplasm into an epithelial cell are seen at arrows. M = mitochondria; ER = endoplasmic reticulum; N = nucleus. (×13,500.)





Figs. 13 and 14 (Pappas and Smelser). Electron micrographs of sections through the basal portion of the pigment cells. The irregular basal surfaces are resting on a basement membrane (BM). Infoldings of the basal surface occur (I). The finely fibrous basement membrane is in close association with the coarse collagen fibrils (C) of the connective tissue stroma. (Figure 13, ×22,000; Figure 14, ×17,600.)

Deep infoldings of the cell membrane, however, do not contain basement membrane material ("I" in figs. 13 and 14). The fine fibrillar material of the basement membrane is intimately related to the more coarse collagen fibrils of the connective tissue stroma. The main differences between the basement membrane fibrils and the thicker collagen fibrils appear to be those of diameter and periodicity.

Observations on experimentally modified epithelium

Our observations indicate that similar changes are brought about by injecting the eye with mineral oil, and presumably blocking Schlemm's canal, or by systemic injection of Diamox or endotoxin.

An attempt was made to interfere with drainage of the aqueous humor from the eye by adding heavy mineral oil to the anterior chamber. A quantity of aqueous humor was withdrawn and replaced through the same needle by an approximately equal amount of oil. The needle was introduced into the eye at the limbus, at an acute angle to the surface of the globe in an attempt to reduce the escape of the oil through the injection site. The oil was placed in the anterior chamber; at autopsy none was found in the posterior chamber. The oil injection preceded autopsy by approximately 20 hours. When the animals were killed, considerable variability was found in the turgor of the globe, and the ciliary processes showed a variable engorgement of their vessels. The intraocular pressure of some of the eyes was actually lower than the controls at the time of autopsy, whereas in others the pressure appeared to be high when tested digitally. Only the latter eyes are reported. In experiments with Diamox, 100 mg./kg. were injected intravenously 45 to 50 minutes prior to autopsy. Four mg./kg. of Shigella endotoxin* was injected intravenously one and one-half hours before autopsy.

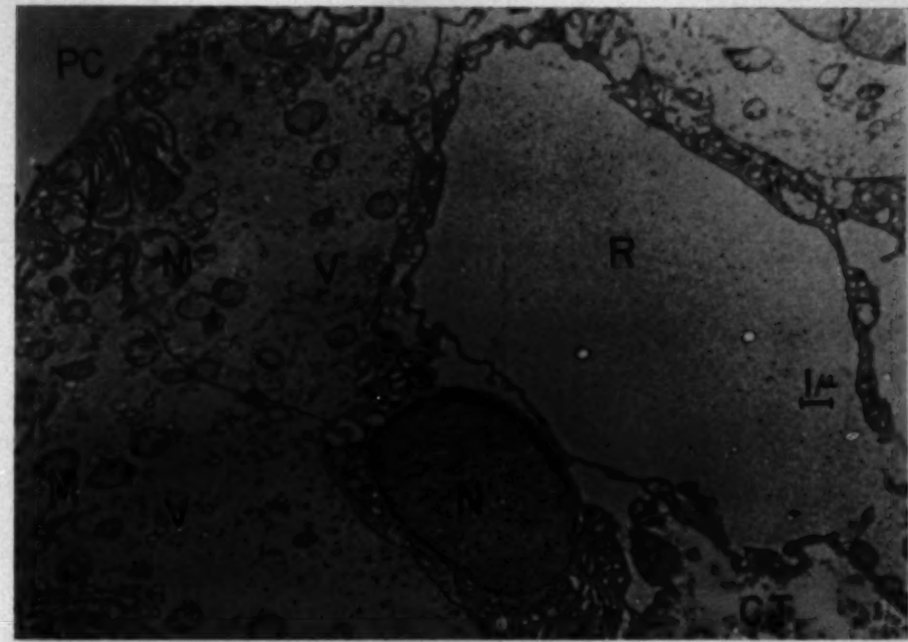
* Shigella endotoxin was kindly supplied by Dr. S. P. Halbert.

The most obvious change found in the mineral oil preparation was the presence of large vacuoles, one to 20 microns in diameter, in the cytoplasm of the pigment cells (fig. 15). The epithelial cells facing the posterior chamber enlarged, their mitochondria became swollen, and many vesicles were found in the cytoplasm (figs. 15 and 16). The vesicles ranged in size from 250 Å to 3,000 Å in diameter. The interdigitations between the cells largely disappeared (figs. 15 and 16), as did the infoldings of the free surface of these cells to some extent. In addition to the large vacuoles, small vesicles in the size range of those found in the surface cells were also found in the pigment cells, but in these cells they appeared to be swollen elements of the endoplasmic reticulum (fig. 17). At the arrows in Figure 17, a vesicle continuous with the outer nuclear membrane is seen. Large fluid-containing spaces (P) were found between the pigment cells as well as between the two cell layers (fig. 17).

Similar results were obtained after intravenous injection of Shigella endotoxin. The Shigella endotoxin produced a slight hyperemia of the iris and a marked flare in the aqueous humor, indicating that large quantities of protein had passed the blood aqueous barrier.

Large fluid-containing spaces between the two epithelial cell layers were found (V_1) as well as large vacuoles within the cytoplasm of the pigment cells (V_2) (fig. 18). The interdigitations between the adjacent epithelial cells and most of the surface infoldings largely disappeared. The cytoplasm of these cells was filled with vesicles (fig. 20). Rows of vesicles (fig. 19) are probably formed by the pinching-off of the cell membrane infoldings. Similar rows of vesicles can be found in normal cells, but to a much smaller degree.

Diamox treatment produced results somewhat similar to the above. Forty-five to 50 minutes after an intravenous injection of Diamox the ciliary processes were fixed and



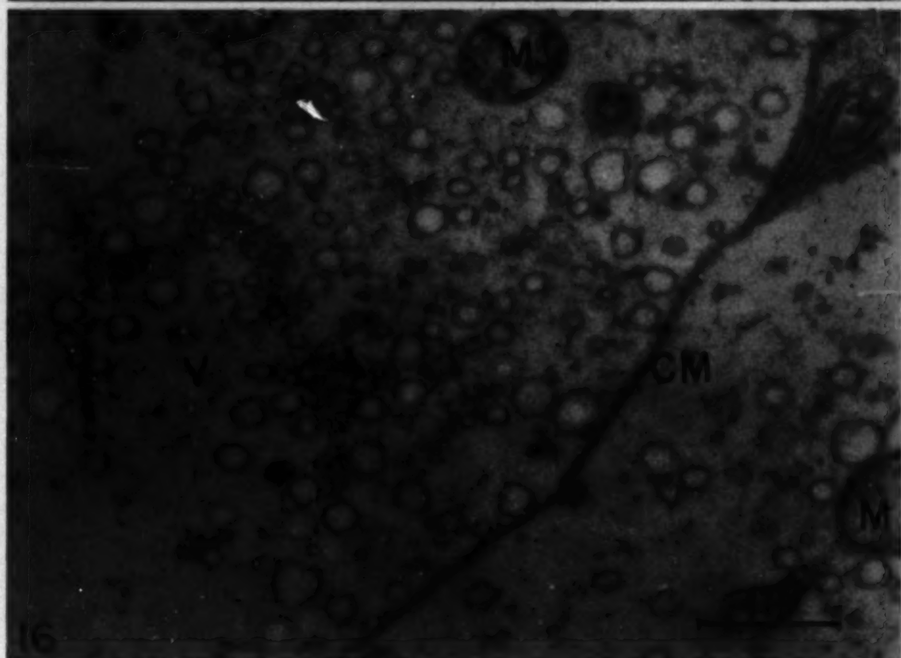
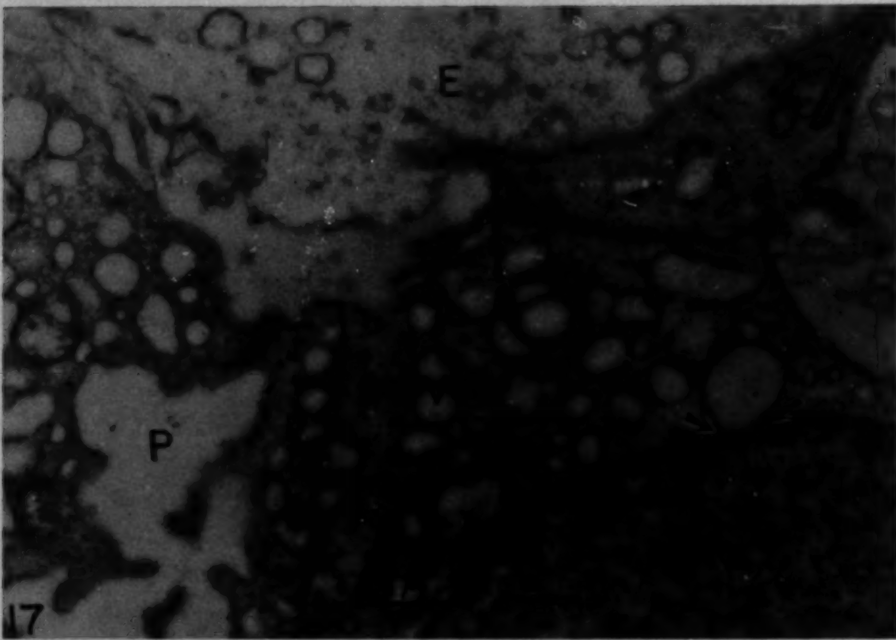


Fig. 15 (Pappas and Smelser). Electron micrograph of a section of ciliary epithelium taken after aqueous humor drainage was blocked with mineral oil. Mitochondria (M) in epithelial cells facing the posterior chamber (PC) are somewhat swollen. Small vesicles (V) are found in the cytoplasm. Extremely large vacuole (R) as well as smaller vacuoles are found in the pigment cell adjacent to the connective tissue stroma (CT). Nucleus (N) of pigment cell is seen. (×4,800.)

Fig. 16 (Pappas and Smelser). Preparation similar to that of Figure 15. Vesicles (V) are found throughout the cytoplasm. The vesicles range in size from 250 to 3,000 Å. The cell membranes (CM) show less of the elaborate interdigitations seen in normal cells. Mitochondria (M) are somewhat swollen. (×21,500.)



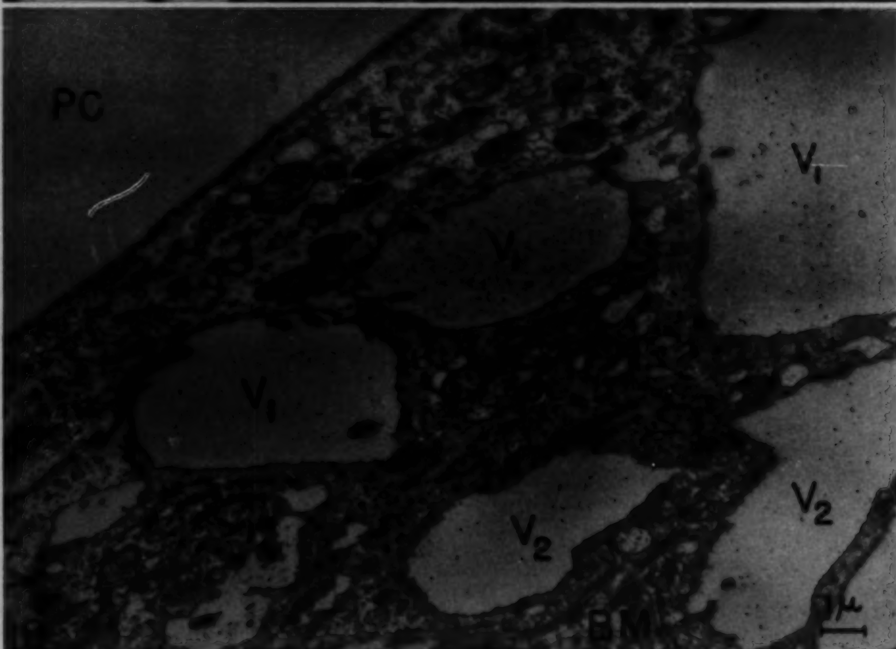
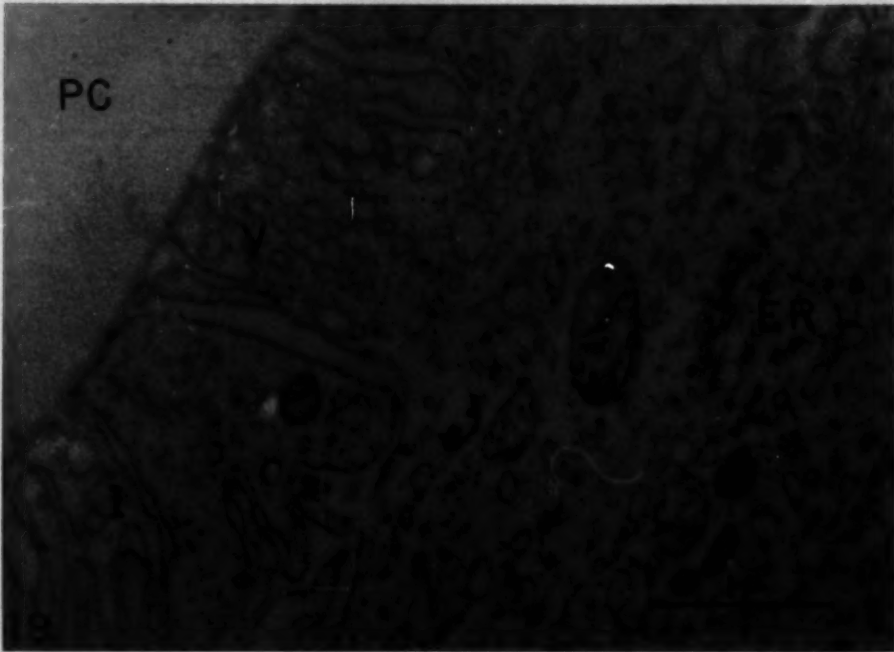


Fig. 17 (Pappas and Smelser). Preparation similar to that of Figure 15, showing vesicles (V) in the cytoplasm of a pigment cell. These vesicles appear to be swollen elements of the endoplasmic reticulum. At arrows the outer nuclear membrane of the nucleus (N) is continuous with a vesicle. Large fluid-filled spaces between adjacent pigment cells are found (P) as well as between ciliary epithelium (E) and the pigment cells. (×21,500.)

Fig. 18 (Pappas and Smelser). Electron micrograph of a section of ciliary epithelium taken one hour after an injection of *Shigella* endotoxin. Large fluid-containing spaces (V_1) are found between the ciliary epithelial layer (E) facing the posterior chamber (PC) and the pigment cell layer (P) resting on a basement membrane (BM). Large vacuoles (V_2) are found in the cytoplasm of the pigment cells (×7,000.)



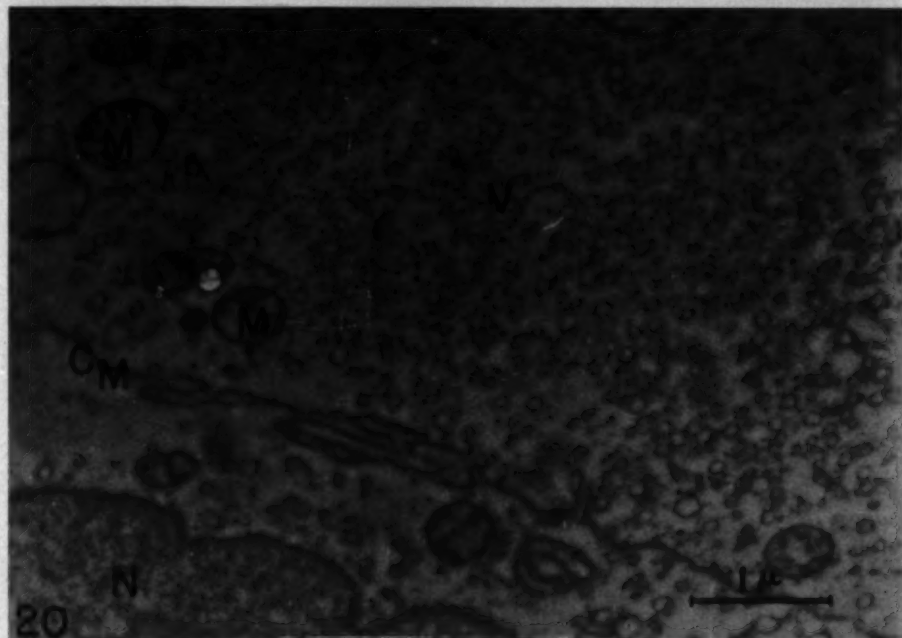


Fig. 19 (Pappas and Smelser). Preparation similar to that of Figure 18. Ciliary epithelial cell facing posterior chamber (PC) has infoldings of its surface (I). The arrangement of vesicles (V) in rows suggests that vesicles are formed by the pinching-off of the surface infoldings (I). Mitochondria (M) and elements of the endoplasmic reticulum (ER) are found in the cytoplasm. (×28,800.)

Fig. 20 (Pappas and Smelser). Cytoplasm of ciliary epithelium filled with vesicles (V) after endotoxin injection, similar to mineral oil experiments (see fig. 16). Slightly swollen mitochondria (M) are also found, as well as somewhat straightened out cell boundaries (CM). (×21,500.)

examined in the electron microscope. The presence of vesicles arranged in linear fashion along an area of interdigitation and infoldings can be seen at arrows in Figures 21 and 22. The long straight rows of vesicles suggest that they are formed by the pinching-off of the interdigitating membranes and surface infoldings.

The cytoplasm was filled with vesicles; however, the very large vacuoles found in the cytoplasm of the pigment cells or between the two cell layers after mineral oil or endotoxin treatment were not found after Diamox injection. Furthermore, the disappearance of the surface infoldings (fig. 22), as well as the interdigitations between adjacent cells, was not as marked after Diamox treatment.

Discussion

Accommodation is achieved by the contraction of smooth muscle in the ciliary body. The muscle fibers are fastened to an endomysium, which is probably similar to that of striated muscle fibers invested with the reticular fibers of the sarcolemma (the myotendon junction) as described by Porter.[14] The collagenous fibers of the endomysium form a loose network in the connective tissue area. The fine fibrillar material of the basement membrane is in intimate association with the collagen fibers (figs. 13 and 14). The basement membrane, in turn, is in close association with the highly irregular contours of the basal portion of the pigment cells. The intimate association of the basement membrane with the irregular basal surface of the cells probably results in the close adhesion of the connective tissue elements to the pigment cell layer. The two layers of epithelium of the ciliary processes represent the forward continuation of the retina with its pigment epithelium. These two layers closely adhere to each other, due mostly to the presence of the "adhesion plates" or desmosomes, but also to small fingerlike projections of pigment cell processes into the basal portion of the epithelial cells (figs. 11 and 12). This is interesting because the two layers arise separately and come in contact with each other as a result of folding during embryologic development. The mode of "attachment" found here is found elsewhere between similar or sister cells which have been produced by mitosis.

In examining nonsectioned fragments of zonular fibrils, Ruska and Schwarz[15] reported a periodicity of about 700 Å, similar to that of collagen. In sectioned material Böke and Lindner[16] verified these findings. However, Zanella[17] found, in addition to 600 to 700 Å fibrils, other small ones without apparent periodicity. In our preparations, we have observed a periodicity of 80 to 110 Å.

Zonular fibers do not penetrate into or between the epithelial cells facing the posterior chamber, but rather terminate in and form the internal limiting membrane. This "membrane" represents a condensation of zonular fibrillar material (figs. 6, 7, and 8). It does not appear that the internal limiting membrane is attached in any way to the cell membrane of the epithelial cell. The surface of the ciliary processes is very irregular, and the internal limiting membrane follows these irregular surface contours very closely. The method of attachment of the zonular fibers to the ciliary epithelium may be considered similar to the "attachment" of a tight-fitting glove on one's hand. The mere close approximation of the two membranes—the epithelial cell membrane and the internal limiting membrane—on an irregular contour would give a very close, tight "attachment" of the zonular material. This is essentially the situation which exists at the myotendon junction.[14] Thus the ciliary process is a securely integrated unit for reflecting the pull on the zonule by the smooth muscle cells located in the connective tissue stroma of the ciliary body.

The characteristic elaboration of the cell membrane into infoldings and intricate interdigitations in the normal surface ciliary epithelial cells shown in these micrographs

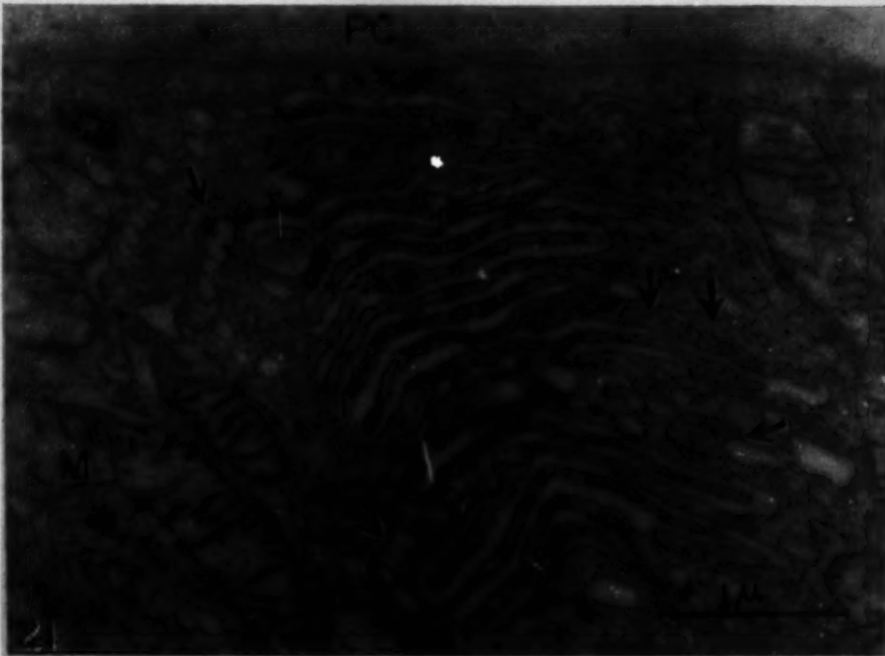


Fig. 21 (Pappas and Smelser). Electron micrograph of a section of ciliary epithelium taken 45 minutes after Diamox injection. Elaborate interdigitation between a portion of two ciliary epithelial cells facing the posterior chamber (PC) is found. At arrows the arrangement of vesicles in long straight rows suggests that vesicles are formed by the pinching-off of the interdigitating membranes. Mitochondria (M) are somewhat swollen. (×29,300.)

Fig. 22 (Pappas and Smelser). Preparation similar to that of Figure 21. A portion of a ciliary epithelial cell facing the posterior chamber (PC) is seen. At arrows, long rows of vesicles are found suggesting that these vesicles are also formed by the pinching-off of the surface infoldings (I). (×36,000.)

agrees with recent reports by Holmberg.[3,5] The fine structure of the cytoplasm with its organelles—mitochondria, endoplasmic reticulum, and Golgi complex—has also been described extensively by Holmberg.[5]

When the blockage of aqueous humor drainage by mineral oil caused an increase of intraocular tension, marked changes in the fine structure of the ciliary epithelium appeared. These changes may be summarized as follows:

1. A large accumulation of vesicles throughout the cytoplasm of the ciliary epithelial cells is noted.

2. The elaborate interdigitations between the epithelial cells and the infoldings on the surface become less evident.

3. The arrangement of rows of vesicles at sites where interdigitations and infoldings are usually found indicates that the vesicles may be formed by the pinching-off of these membranes.

4. Large fluid-containing spaces between the two cell layers may be found, as well as large vacuoles in the cytoplasm of the pigment cells.

5. Elements of the endoplasmic reticulum become swollen in the cytoplasm of the pigment cell, and to a lesser degree in the surface epithelial cells.

6. Mitochondria in both cell layers become somewhat swollen.

After a single intravenous injection of Shigella endotoxin, the aqueous humor became cloudy, indicating the presence of large amounts of plasma protein. The changes in the fine structure of the ciliary epithelium appeared to be similar to those described above after mineral oil treatment.

Injection of Diamox produced less drastic, yet similar results. Large fluid spaces between the two cell layers were not found, nor were large vacuoles seen in the cytoplasm of the pigment cells. The interdigitations and infoldings were more evident after Diamox treatment than after mineral oil or endotoxin treatment. However, even after Diamox, the infoldings and interdigitations were less evident than in the normal cells.

Holmberg[4] has suggested that the presence of vesicles after Diamox treatment is due to the breakdown of the Golgi complex which is normally found in the basal portion of the ciliary epithelial cells. However, the ciliary epithelial cells are relatively poor in Golgi material as shown in our light and electron microscope preparations. The amount of Golgi material does not appear sufficient to account for the large accumulation of vesicles after treatment. Another, perhaps more abundant, source of vesicles may be the breakdown of the infoldings and interdigitations of the cell membrane. The disruption of normal function of the ciliary epithelium in the production of aqueous humor, regardless of how it is accomplished—(whether by blockage of the drainage angle, or by endotoxin or Diamox treatment)—produces similar morphologic results.

A characteristic of cells engaged in fluid transport is the elaboration of their cell membranes.[6] The basal portion of the proximal convoluted tubule cells of the kidney shows a high degree of infoldings.[18] The basal portion of the ependymal cells of the choroid plexus displays many infoldings of the membrane.[19] It appears likely that large increase in cell membrane area by the elaboration of infoldings indicates high secretory activity of the cell. Therefore, the loss of the cell membrane infoldings and interdigitations of the ciliary epithelial cells may be considered a morphologic indication of disruption of normal aqueous humor secretion.

Summary

1. The epithelial cells facing the posterior chamber are characterized by extensive infoldings and elaborate interdigitations of their margins.

2. The internal limiting membrane (150 to 300 Å thick) is in close apposition to the contours of the cell surface, but does not line the infoldings and interdigitations of these cells.

3. Zonular fibers do not penetrate into or between the epithelial cells but rather termi-

nate in and form the internal limiting membrane.

4. The structural cohesiveness of the various parts of the ciliary processes (that is, zonule, the two cell layers, the basement membrane, and the connective tissue stroma) as a significant factor in accommodation is discussed.

5. The disruption of normal function of the ciliary epithelium in the production of aqueous humor—regardless of how it is accomplished (whether by blockage of the drainage angle with mineral oil, or the systemic injection of Shigella endotoxin or Diamox)—produced similar morphologic results. Diamox brought about the least drastic changes.

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References

1. McCulloch, C.: The zonule of Zinn: Its origin, course, and insertion, and its relation to neighboring structures. Tr. Am. Ophth. Soc., **52**:525-585, 1955.

2. Friedenwald, J. S.: The formation of the intraocular fluid Am. J. Ophth., **32**:9 (June Pt. II) 1949.

3. Holmberg, Å.: Studies of the ultrastructure of the non-pigmented epithelium in the ciliary body, Acta Ophth., 33:377, 1955.

4. ———: Changes in the ultrastructure of the ciliary epithelium during inhibition of the secretion of aqueous humour. In Electron Microscopy, Proc. Stockholm Conference. Academic Press, New York, 1956, p. 139-143.

5. ———: Ultrastructural changes in the ciliary epithelium following inhibition of secretion of aqueous humour in the rabbit eye. Stockholm, 1957.

6. Pease, D. C.: Infolded basal plasma membrane found in epithelia noted for their water transport. J. Biophys. & Biochem. Cytol., **2**(Suppl.)203-208, 1956.

7. Moore, D. H., and Grimley, P. M.: Problems in methacrylate embedding for electron microscopy. J. Biophys. & Biochem. Cytol., **3**:255-260, 1957.

8. Palade, G. E., and Porter, K. R.: Studies on the endoplasmic reticulum. I. Its identification in cells in situ. J. Exper. Med., **100**:641, 1954.

9. Dalton, A. J., and Felix, M. D.: A comparative study of the Golgi complex. J. Biophys. & Biochem. Cytol., **2**(Suppl.):79-84, 1956.

10. Watson, M. L.: The nuclear envelope: Its structure and relation to cytoplasmic membranes. J. Biophys. & Biochem. Cytol., **1**:257, 1955.

11. Porter, K. R.: Observations on the submicroscopic structure of animal epidermis. Anat. Rec., **118**:433, 1954.

12. Odland, G. F.: The fine structure of the attachment between cells in human epidermis. Anat. Rec., **130**:349-350, 1958.

13. Fawcett, D. W.: Observations on the cytology and electron microscopy of hepatic cells. J. Nat. Cancer Inst., **15**:1475, 1955.

14. Porter, K. R.: The myo-tendon junction in larval forms of *Amblystoma punctatum*. Anat. Rec., **118**:342, 1954.

15. Ruska, H., und Schwarz, W.: Elektronenmikroskopische Untersuchungen am Ligamentum suspensorium lentis des Rinderauges. Z. wiss. Mikrosk., **60**:181-188, 1951.

16. Böke, W., und Lindner, E.: Elektronenmikroskopische Untersuchungen an Zonulafasern. Arch. f. Ophth., **157**:101-106, 1955.

17. Zanella, E.: Inadagini elettroniche sulle fibre della zonula dello Zinn. L'ateneo Parmense, **27**:1-8, 1956.

18. Rhodin, J.: Correlation of ultrastructural organization and function in normal and experimentally changed proximal covoluted tubule cells of the mouse kidney. Stockholm, 1954.

19. Maxwell, D. S., and Pease, D. C.: The electron microscopy of the choroid plexus. J. Biophys. & Biochem. Cytol., **2**:467-474, 1956.

Discussion

Dr. V. Everett Kinsey (Detroit): I would like to congratulate the authors on a splendid paper both from the standpoint of morphology and from that of aqueous humor dynamics.

The first comment I should like to make is in connection with the aqueous humor dynamics. Recently, we have been measuring the turnover rate of sodium and chloride in the posterior and anterior chamber. From the data obtained, the fraction of each ion which enters by diffusion and that which enters in the fluid secreted by the ciliary process has been calculated. In the case of sodium, about one third enters by diffusion and two thirds by secretion; chloride, on the other hand, enters mostly by diffusion (70 percent).

The relevance of this paper to these findings is that the photographs permit us to speculate as to where in the ciliary epithelium these two processes

occur. For instance, the large area of interdigitation between the cells would facilitate diffusion and one could agree with the implications made by the authors that the greater portion of the transfer may well occur between the cells. The vacuoles within the cells may contain the "secreted fluid" which may be transferred into the posterior chamber by pinocytosis.

Concerning the action of Diamox, I think I would be inclined to agree with the authors' interpretation. It looks as though the surface membrane is broken so that there is much less of it. With less membrane, one would expect less efficient formation of aqueous.

DR. LORENZ E. ZIMMERMAN (Washington, D.C.): I, too, would like to congratulate these workers on the magnificent pictures they have shown here, and also over at the exhibit. I would like to ask three questions.

First, with regard to the insertion of the zonule. Studying the pictures at the exhibit, I thought the fine zonular collagenlike fibers in many of the pictures seemed not to stop at the outer limiting membrane but passed through and inserted into the cell membrane itself. I would like to have further comments as to why the authors believe they do not insert into the cell.

A number of their pictures seemed to me to be so beautifully illustrative of the fact that they did go into the cell membrane. It seemed as if there were little whiskers sticking out from the cell itself.

The second question concerns the electron density of the "pigment epithelium" in these albinos. Do you have any clues as to what it is that makes the cytoplasm of these cells appear more dense with a number of different histologic techniques, including those of electron microscopy?

The third question is in regard to the little projections that you illustrated from the pigment cell into the inner cell. Is this analogous to the villouslike projections that one sees in the retinal pigment epithelium, as is shown so beautifully in Dr. Garron's exhibit? They seem to be so very small by compariosn here in the ciliary region.

DR. BERNARD BECKER (St. Louis): I, too, enjoyed this paper, and want to make one comment and ask a question.

It seems that the authors have provided us with one of the controls that Holmberg did not include. Holmberg used as a control for Diamox a sulfonamide that is not a carbonic anhydrase inhibitor. The important control he did not report upon was the effects of other means of altering intraocular pressure. The question arises as to whether the appearance of increased vesiculation following Diamox, as noted by Holmberg, follows the lowering of intraocular pressure per se. In the present work an example is presented of increased vesiculation when intraocular pressure is raised by mineral oil, and this therefore is an important control.

My question is one of interpretation. Holmberg, as I understand it, was inclined to believe that vesicles were present normally in the ciliary epithelium, and that the increased vesiculation could be accounted for on the basis of a slower transport of these vesicles. I think he was largely influenced by the work of Bennett and others on pinocytosis as a mechanism of ion transport. I would like to ask the authors whether they think this is as feasible as their explanation for the appearance of more vesicles in the cell.

DR. GEORGE D. PAPPAS (closing): First, pertaining to Dr. Zimmerman's question on the insertion of the zonule:

When the internal limiting membrane is very close to the cell membrane it is indeed very hard to see whether the fine zonular fibrils terminate and form the internal limiting membrane. However, sometimes when the internal limiting membrane has a larger gap, say about 200 Angstroms, between itself and the cell membrane, then we can see very clearly that the zonular material does terminate and form the internal limiting membrane.

As to why the pigment epithelium is dense, we don't know. I would say that in studying the normal albino material there is much more of the endoplasmic reticulum, with its associated granules, present in the pigment cell than in the epithelial cell.

As for the projection of pigment cells and whether they are analogous to the villi, perhaps they are; but I think they are more analogous to the little projections or "collar buttons" that Dr. Faucett has described between liver cells.

Dr. Becker asked if the vesicles are normally present. Yes, they are in the cytoplasm of these cells. Not only that, but in normal preparations we can make out, once in a while, rows of these vesicles suggesting that they are formed by the breakdown of the elaborated cell membrane. The interdigitations and infoldings are not static structures. When we fix them we preserve the shape. If we fix them two or three minutes later the contours would be different. The infoldings and the interdigitations are a very dynamic labile system.

As for pinocytosis, yes indeed. With the work of Palade we know most cells engage in this activity, but it is a little too early to say what role pinocytosis plays in aqueous humor dynamics, what direction these vesicles are moving in, or anything of that sort. There are vesicles normally present, but not as many as we find in our experimental conditions.

With that I would like to close and say that when these cells appear "normal," that is, when they appear like other cells, more-or-less rectangular and cuboidal with even limiting membranes, perhaps at that time they are not engaged in the process of aqueous formation, and that aqueous formation is morphologically reflected in the elaboration of the cell membranes. Indeed, Dr. Pease has noted that epithelial cells elaborated for water transport have infolded plasma membranes.

THE APPLICATION OF ULTRASONICS LOCATING TECHNIQUES TO OPHTHALMOLOGY*

HEORETIC CONSIDERATIONS AND ACOUSTIC PROPERTIES OF OCULAR MEDIA: PART I. REFLECTIVE PROPERTIES

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Pleasantville, New York

PART I: THEORETIC CONSIDERATIONS

An ultrasonic locator is a device which combines the methodology of radar and sonar for the acoustic visualization of tissue.

The operation of the ultrasonic locator simulates the slitlamp except that a pulsed ultrasound beam is substituted for the light beam. High frequency acoustic waves travel in straight lines (subject to refraction and diffraction limitations) and exhibit many of the properties of light. When an ultrasound pulse crosses an interface between tissues of different acoustic properties, a portion of the energy is reflected and the rest propagates into the deeper tissues. The reflected acoustic energy is picked up by a suitable microphone or transducer, which converts it into an electrical impulse of proportional energy. Using standard radar techniques, the electric impulse can be displayed as a point of light on a cathode ray tube screen by intensity modulation. The intensity of the light varies with the strength of the reflected energy (fig. 1-A). When the receiving-transmitting system is moved so as to scan the tissues, and the display trace is suitably co-ordinated therewith, the summation of the points of light forms an acoustic image (echogram or sonogram) of the tissues under study (fig. 1-B).

Another method of presenting the electric impulses from the transducer is to represent them in a time-amplitude relationship in which the distance along the horizontal axis represents the time required for the sound to travel from the transmitter to the tissues and back, hence range or depth, and the vertical displacement represent the strength of the reflected impulse (fig. 1-C). This simpler method of representation although sacrificing two-dimensional visualization is of value for quantitative measuring purposes.

Ultrasonic energy is capable of injuring an eye just as is any other form of radiant energy. Therefore, the upper limits of both the average and peak energy of the transmitted energy incident on any critical tissue must be below the levels at which permanent injury can be produced. Within this limitation ultrasonic visualization of the eye is possible if the portion of the energy absorbed and scattered is small enough, and the strength of the reflection is large enough to permit an echo to be detected at the transducer. These factors must be considered in relation to the power level of the transmitted pulse and the minimum detectible signal level of the transducer and its associated amplifier.

For example, assume that the transducer and amplifying system can just detect a signal 120 decibels (db.) smaller than the transmitted pulse. (120 db. represents a power ratio of 1,000,000,000,000 and an amplitude ratio of 1,000,000). If on the path into the eye, the combined absorption,

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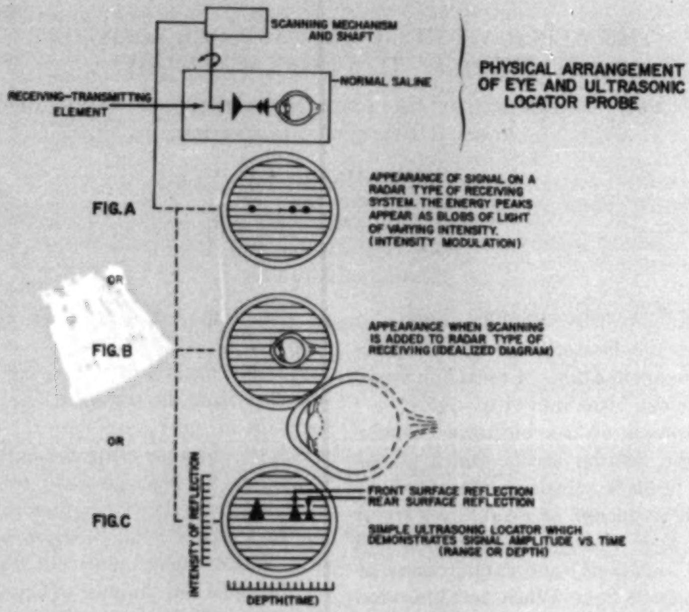


Fig. 1 (Baum and Greenwood). Various methods of showing information obtained by ultrasonic localization.

reflection, and scattering loss of the cornea is 5.0 db., of the lens is 20 db., and of the vitreous is 5.0 db., then a signal 30 db. below the transmitted signal will be incident on the retina. If the reflection from the retina is 40 db. below the signal incident on it, and the same 30 db. loss occurs on the path back through the eye, then the signal incident on the transducer will be 100 db. below the transmitted signal, and will therefore be 20 db. stronger than the minimum detectible signal.

Because of these limitations, measurement of the acoustic properties of the eye at the frequency at which we proposed to operate (15 mc.) are essential for proper interpretation of echograms. These data are to the best of our knowledge not available any where else.

This paper will be limited to a description of the technique of measuring the acoustic reflection of ocular tissues, an analysis of the factors affecting reflection, and a listing of the reflection factors of various eye tissues.

The absorption coefficients of the ocular tissues will be reported in a separate paper. Both the reflection and absorption values will then be combined and a theoretic composite echogram of the eye will be constructed. The theoretic composite echogram will then be compared with actual interface reflections.

Acoustic reflection theory

Acoustic interface reflection is analogous to optical interface reflection; that is, as sound passes from a medium of a given acoustic impedance to a medium having a different acoustic impedance a portion of the energy is reflected at the interface. (The same phenomenon occurs when light passes through substances possessing different indices of refraction, fig. 2).

The strength of the acoustic reflection depends mainly upon the difference of acoustic impedances at the interface of the adjacent tissues.

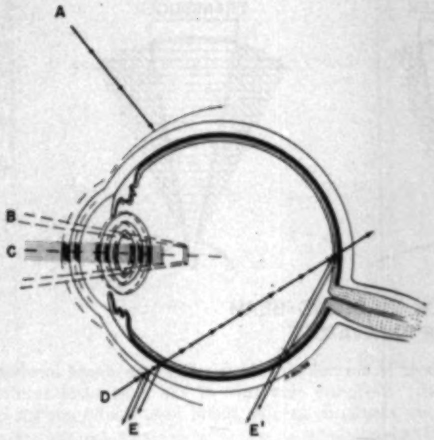


Fig. 2 (Baum and Greenwood). A comparison of the visualization of the eye by the light and sonic slitlamps.

(A) Light incident upon the heterogenous surface of the sclera is absorbed, diffused, and reflected so that only the surface of the sclera can be seen.

(B) Light incident upon the homogenous cornea is transmitted into the depths of the eye.

(C) As light passes through zones of optical discontinuity, that is, different indices of refraction, a reflection occurs at each interface.

(D) High frequency sound waves can penetrate the soft tissues of the body regardless of their optical properties.

(E and E') As the sound waves pass through tissues possessing different acoustic impedances, reflections are set up at each interface. These reflections are detected by the receiving crystal and converted into electric impulses. The latter are amplified and then converted into light of varying intensities, so as to form a picture on the face of the cathode ray tube.

Equations used to determine acoustic impedance and reflection

The acoustic impedance of a non-lossy substance is the product of the density of the substance and the velocity of sound in the substance, that is

$$z = vd \quad (1)$$

where z = acoustic impedance
v = velocity of sound in the substance
d = density of the substance

The strength of the reflection at an interface may be calculated by Equation 2, if the acoustic impedances of the adjacent substances are known. Conversely if the reflection factor and the acoustic impedance of one substance are known, the acoustic impedance of the other substance can be calculated.

$$r = \frac{z_2 - z_1}{z_2 + z_1} \quad (2)$$

where:
r = coefficient of reflection (amplitude)
r^2 = coefficient of reflection (power)
z_1 = acoustic impedance of first substance
z_2 = acoustic impedance of second substance
$20 \log_{10} r = r_{db}$ = db ratio of reflected power to incident power

The equation shown above assumes loss free media. However, substances such as tissues, rubber, and some plastics, do not conform strictly to Equation 1, because they possess a reactive component of acoustic impedance, representing absorption. The impedance of such substances is shown by:

$$Z_1 = \frac{dv}{1+a^2} + i\,\frac{adv}{1+a^2} \quad (3)$$

where:
Z_1 = acoustic impedance of "lossy" material
d = density of the material
v = sound velocity in the material

$a = \frac{\alpha v}{\omega}$ = loss parameter

α = sound attenuation in material (Nepers/cm.)
$\omega = 2\pi f$
$i = \sqrt{-1}$

The attenuation, α, is very low in most elastomers at frequencies below 100 kilocycles. However, α increases with increasing frequency, and becomes appreciable in some materials at frequencies above 500 kilocycles. Equation 2 is valid for both loss-free and lossy substances.

Factors which modify reflection

1. *Temperature.* The velocity of sound in a substance and its density are in general temperature dependent. Altering the temperature of the coupling media may alter the acoustic impedance of one or both of the substances at the interface and hence the reflection (equation 1).

2. *Osmotic effect.* Experimental data indicate that the acoustic impedance of dead and

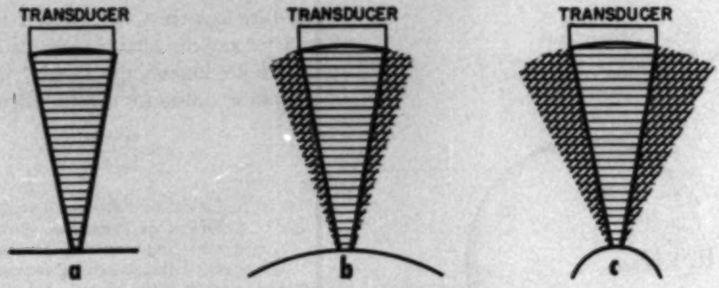


Fig. 3 (Baum and Greenwood). (a) When transducer is normal to a flat surface, the sound incident upon the glass plate is reflected to the transducer. (b) Moderate curvature of the glass plate results in some loss of sound energy. (c) Increasing curvature results in greater sound loss. Sound energy is lost due to curvature of glass plate or tissue.

injured living tissues varies with the osmotic pressure of the solution they are suspended in. This observation requires further study for complete validation.

3. *Lossy substances.* The reflection from an interface between lossy substances is determined by equations 2 and 3. Using constants measured for ocular tissue it is computed that the reactive term changes the theoretic reflections by less than 0.5 db. This is too small to be measured with our equipment. Furthermore, it is presumed to be constant for a given tissue interface and its effect would therefore be insignificant for practical measurement. Equations 1 and 2 may therefore be used in place of Equations 2 and 3.

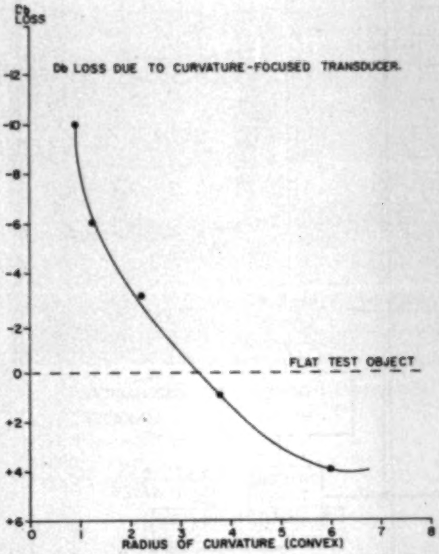
Sound beam shape and sensitivity

The beam is sharp enough to resolve objects less than 0.5-mm. apart. Uniform sensitivity less than ± 1.0 db. variation holds only over a 6.0-mm. range. To obtain comparative or reproducible readings the test object must be within this 6.0-mm. zone. Fortunately, it is very simple to localize this position by using both the intensity and amplitude modulated oscilloscopes.

Effects of radius of curvature

Ultrasound obeys the same geometric laws of reflection as light, that is the angle of incidence equals the angle of reflection. Therefore, when the sound beam strikes a curved surface a portion of the energy may be reflected away from the transducer (fig. 3). The amount of energy dissipated by such reflection is a function of the radius of curvature of the test object and the parameters of the sound beam. Because the sound beam cannot be reduced to a point, such losses are present even when the sound beam is normal to the surface being measured. However, at the normal position, there is maximum return of sound energy to the transducer. Graph 1 demonstrates the experimentally determined effects of curvature on echo intensity for the transducer used in subsequent measurements. Convex surfaces produce a loss of sonic energy, except in the zone where the radius of curvature of the examined object coincides with the radius of curvature of the transducer lens. Since this radius usually lies well outside the range of those usually encountered in the eye, this additive factor will be of no concern.

The effect of curvature on reflection is apparently a function of the surface roughness of the sample. The data of Graph 1 were taken with slightly roughened glass surfaces. Perfect optical surfaces do not show any curvature effect in this range of radii of



Graph 1 (Baum and Greenwood). Experimentally determined effects of curvature on echo intensity.

curvature. Since the tissues present a roughened interface, loss due to curvature must be considered in tissue measurements.

Part II: Technique of determining the acoustic properties of ocular structures

A. Method

Description of apparatus. Figure 4 is a block diagram of the major components of an ultrasonic locator. For simplicity, the power supplies have been omitted. Figure 5 is a photograph of the entire unit. Figure 6 is a detailed view of the major components of the receiving and transmitting system. The display components have been removed. Figure 7 is a photograph of the scanning unit. The transducer is submerged in water, or normal saline, which acts as the coupling medium.

B. Technique

Reflection measurements were performed by using the locator as an echoscope (fig. 7). The transducer-tissue relationship was adjusted to yield a maximum signal. Amplifier gain was adjusted so that the signal was well below the amplifier saturation level. A glass block (which yields excellent and known ultrasonic reflection) was substituted for the tissues under test and adjusted for a maximum echo. With the glass block in place, attenuation was introduced by an attenuator box until the signal equalled that received from the tissue under test. The number of decibels of attenuation required to lower the strength of the reflected signal to that from the eye tissues represented the strength of the signal from the tissue, as compared to the reflection from glass, and is readily converted to an absolute value.

The exact site of the echo was determined by using the radar PPI oscilloscope. The normal tissue-transducer relationship was determined by introducing attenuation until only the maximum signal remained visible on the radar scope. This was rechecked to determine if it represented the geometric normal. Location at the focal zone of the sound beam was achieved by an optical system and confirmed by distance measurements with the (A) or amplitude modulated oscilloscope. Only after such positioning were the measurements described above made. The tissues and glass test block were measured in a normal saline coupling medium. The temperature was maintained at a constant value. To obtain comparable values it is of paramount importance that the techniques used for positioning the tissues normal to the transducer and at the zone of maximum and uniform sensitivity be strictly adhered to. All the other factors affecting acoustic reflection must be fully controlled, or unreliable, variable measurements may result.

Standardization of reflection techniques

To determine the validity of the reflection measurements made with this system, upon the tissues of the eye, identical tests were performed on substances whose acoustic

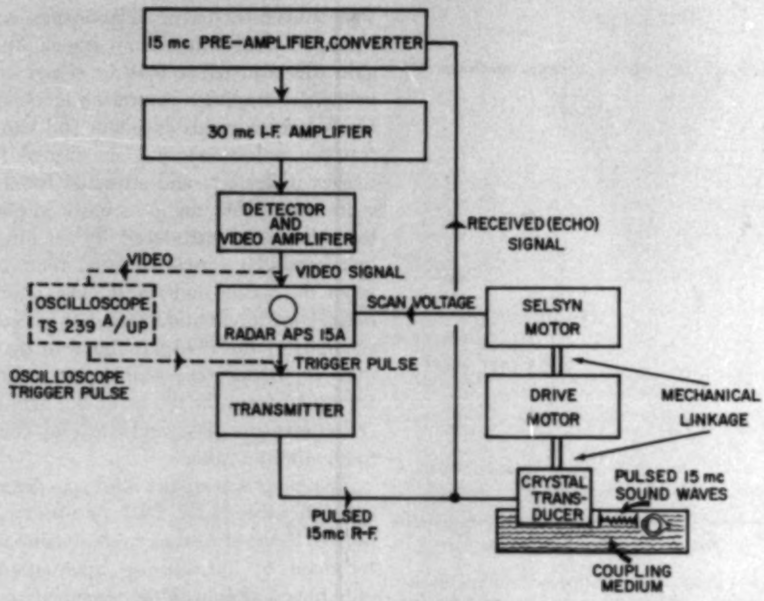


Fig. 4 (Baum and Greenwood). Block diagram of major components of ultrasonic locator.

(A) Pulsed 15 mc. RF current generated by transmitter.

(B) Crystal converts electrical energy into sound. Sound waves travel through water and strike the tissue. Reflected sound waves received back at crystal which reconverts the sound back into electric impulses.

(C) Electric impulses are amplified by receiver.

(D) Radar receiver converts electric impulses into blobs of light of proportional intensity and correct position.

properties are known or can be measured directly, for example, aluminum, tetrachlorethylene, chloroform, and carbon tetrachloride.

The density and the velocity of sound in the test substances were determined. From this the theoretic acoustic impedance and reflection were determined. The coefficient of

Fig. 5 (Baum and Greenwood). Photograph of complete ultrasonic locator.

(A) Specimen tank with Selsyn motor on top
(B) Attenuator box
(C) Pre-amplifier-converter
(D) I-F amplifier
(E) Radar receiver
(F) Oscilloscope
(G) Transmitter
(H) Low voltage transmitter power supply
(I) High voltage transmitter power supply
(J) Receiver power supplies

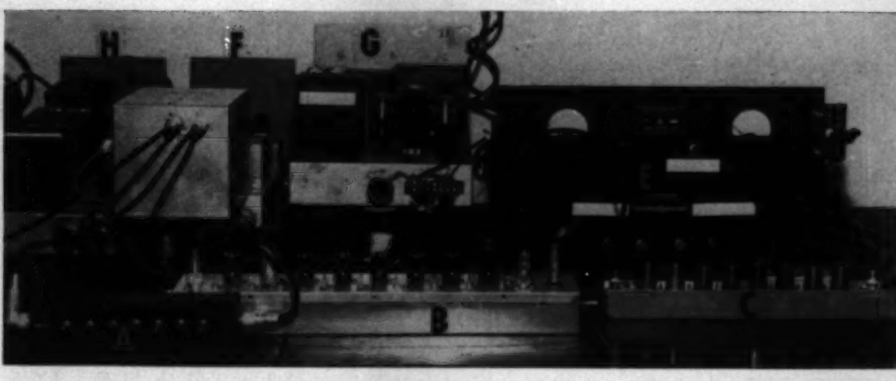


Fig. 6 (Baum and Greenwood). Detailed view of ultrasonic locator.

(A) Attenuator box
(B) Pre-amplifier converter
(C) I-F amplifier
(D) Detector-video amplifier
(E) Receiver power supplies
(F) Transmitter
(G) Low voltage transmitter power supply
(H) High voltage transmitter power supply

reflection at the interface between the test substance and water was then measured with the locator and compared with the theoretic value. Equations 1 and 2 as shown above were used in these determinations (table 1).

Procedure used for measuring the acoustic properties of the eye

The acoustic properties (reflective) of freshly excised beef eyes were measured by the techniques described above. The eyes were first measured along both the anteroposterior and lateral axis, with some attached orbital fat and muscle. These measurements were then repeated on the isolated globe. The eye was then dissected and similar measurements were made of each major structure.

Reflection data beef eye

Table 2 lists the average reflection values for the intact eye and the isolated tissues of the eye, while approximately in their natural state of curvature.

Discussion of reflection data

Where two surfaces could be measured, for example, the cornea, lens, and sclera, the reflection value refers only to the anterior surface. The cornea was measured prior to its removal from the eye so as to retain its curvature. Both the uncorrected and curvature corrected values were given. The radius of curvature chosen is the average between the vertical and horizontal radii of the cor-

Fig. 7 (Baum and Greenwood). Detailed view of scanning system. (A) Selsyn motor—for synchronous positioning of transducer and sweep of cathode ray tube. (B) Drive motor. (C) Transducer, mounted at the end of the shaft of the Selsyn motor. (D) Tank for submerging specimens and transducer.

TABLE 1

STANDARDIZATION OF REFLECTION MEASUREMENTS*

Substance	Measured Values				Standard Values[1]			
	Density	Velocity	Z	Reflection (in db.)	Reflection (in db.)	Z	Velocity	Density
Aluminum	2.69	62.6	17.01	−3.0	−1.52	16.9	62.6	2.71
Naphthalene Monobromide ($C_{10}H_7Br$)				−18.0	−16.8	2.04	13.72	1.487
Carbon Tetrachloride ($C\,Cl_4$) (N.F.)				−43.0	−41.5	1.47	9.21	1.596
Chloroform (U.S.P.)				−42.2	−44.0	1.472	9.86	1.493
Tetrachlorethylene (C_2Cl_4)				−26.0	−24.9	1.67	10.27	1.623

* Air water interface is the reference level.
Density = (gm./cm.^{3}).
Velocity = $10^4\times$(cm./sec.)
Z = dv = $10^5\times$(gm./cm.^{2} sec.)
[1] Bergman, L.: Ultrasonics. Navships 900,167, p. 209 and 402.

nea. The lens and sclera were handled in the same way. A relatively flat portion of the sclera was measured.

The iris was examined by dissecting off the cornea. Considerable variation of reflection was noted over the surface of the iris. An area midway between the sphincter and the root was chosen as a representative site.

The lens was examined while attached to the zonule, but, following a total iridectomy, the central area of the lens was the site of origin of the echo.

The lens was then removed by cutting the zonular fibers. The adhesions between the posterior surface of the lens and the hyaloid membrane necessitated the cutting away of the vitreous from the lens.

The echo from normal vitreous is so small that it cannot be measured with our equipment.

TABLE 2

REFLECTION OF PULSED 15 MC. ULTRASOUND FROM THE BEEF EYE

	Cornea	Iris	Lens	Retina	Choroid	Sclera
No. Samples	7	11	12	10	10	11
Average radius curvature, cm.	1.3× 1.5	Flat	1.3× 1.3	1.3×2.0 2.5	1.3× 2.0–2.5	1.3× 2.0–2.5
Reflection, corrected to absolute*	−29.6	−46.3	−28.9	−37.5	−36.5	−37.6
Standard deviation between samples in ±db.	±2.1	±5.5	±3.0	±6.0	±6.0	±6.0
Reflection, corrected for curvature	−24.2	46.3	23.1	37.0	36.0	37.1
Z tissue / Z saline	1.131	1.010	1.151	1.029	1.032	1.028

All measurements were made in saline.
* Air water interface (total reflection) is the reference level.
Z = acoustic impedance.
The reflections from the vitreous were too weak to measure.

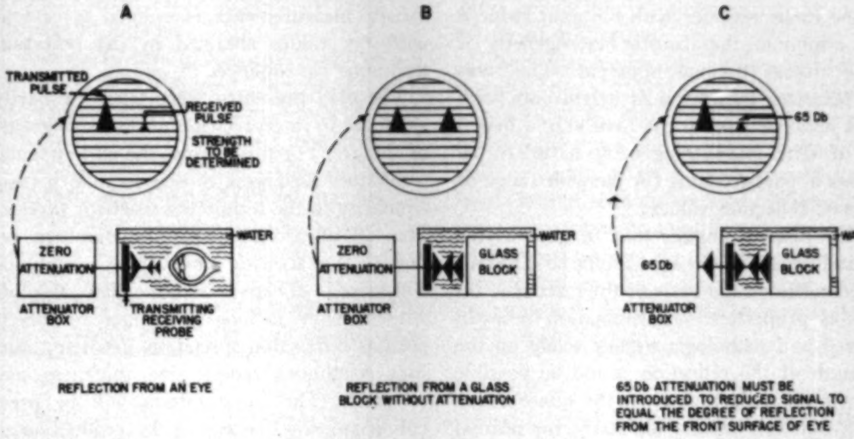


Fig. 8 (Baum and Greenwood). Method of determining reflection from surfaces of eye as compared to reflection from glass. (65 db is an arbitrary figure, actual reflection from the surface of the cornea is −31 db.)

The retinal echo was determined with the vitreous in place, and then after removal of the vitreous. The difference amounted to 1.0 or 2.0 db. which is within the error of measurement. Although some distortion of the radius of curvature did result from the above dissection, handling the eye in this fashion still yielded the geometric form closest to the norm. A retinal area away from the tapetum was chosen, so as to conform more closely to the human eye.

Both the vitreous and retina were then removed and a section of choroid near the optic disc was measured. This area was chosen because it is nearly flat.

The choroid was removed and the sclera in the same area was measured. A significant difference of scleral echo was noted from the choroidal side vs the exterior surface.

Analysis of reflection data

The acoustic impedances of the tissues of the eye as compared to normal saline imply the following:

The lens possesses the highest impedance. This is probably related to its capsule which results in a specular reflection. The cornea is next. It too possesses one of the smoothest surfaces.

The iris comes closest to saline, because of its spongelike structure.

The acoustic impedances of the retina, choroid, and sclera are so close that they cannot be distinguished as separate layers when in normal apposition.

The difference in acoustic impedance between cornea and sclera is in part accounted for by surface roughness and greater variability of measurement for sclera. Lacking a definitive epithelial surface the acoustic interface may lie in its depths.

These points are confirmed by the ultrasonic appearance of the intact eye. The retina or choroid suspended in vitreous can be distinguished because of the difference in acoustic impedance.

Despite meticulous attention to the measuring technique consistent differences were noted both in the intensity of reflection and the wave form from the same structure (for example, retina) with slight variation in the transducer-tissue position. Thus, a narrow range of reflection coefficients for tissues cannot be achieved at this time both because of tissue variability and an inherent error in the measuring technique.

When the structure of the iris, retina, choroid, and sclera were carefully examined

on the radar receiver with the gain reduced to a minimum, the acoustic heterogeneity of these tissues became apparent. This was characterized by a band of bright dots, with black areas between them, instead of a bright line of light. The heterogeneous nature of the tissues in part accounts for the wide range of acoustic reflection values.

This finding implies that in general the normal tissues of the eye cannot be differentiated solely on the basis of their acoustic reflection properties. Discrimination between normal and pathologic tissues solely on the strength of the reflection would be possible only if the reflection from the questionable mass fell well beyond the range for normal tissue, after correction for curvature.

Other criteria for such differentiation have been tentatively established. Sonically empty spaces within a tissue mass or a tissue echo from an abnormal site, for example, the vitreous cavity, are examples of these criteria.

Discussion

To our knowledge the only other paper reporting the use of ultrasonic localization in ophthalmology is that of Mundt and Hughes.[1] Their work was limited to localization by a time-amplitude technique and was conducted at 10 mc.

Higher definition is potentially obtainable at 15 mc. because of the shorter wave length.

Amplitude modulation combined with scanning results in a cross section picture, which makes possible identification of the origin of the echo and indicates the position of the object under study in relation to the surrounding tissues. Without a scanning system it is extremely difficult to determine the exact site from which the echoes arise.

Begui[3] has measured the acoustic properties of the pooled aqueous, vitreous, and calf lenses at 5.0 mc. and 30 mc. Since these measurements were taken at frequences other than 15 mc., the results are not strictly comparable. However, the acoustic impedance of lens determined by Begui, using velocity and density measurements, is in good agreement with the values obtained by the reflection technique we employed.

The data presented were taken primarily as an aid to interpretation of ultrasonograms of the eye. For this reason, the experimental techniques used were chosen because of their similarity to the techniques used for production of ultrasonograms. The numbers reported for acoustic impedances should be considered effective rather than absolute values. They include a number of effects such as diffraction spreading, geometry, surface roughness, sample size, thickness, and so forth. The same effects will be present in varying degrees in the production of actual ultrasonograms. The effective values reported should be more satisfactory than absolute values in predicting and interpreting echo amplitudes to be expected in practical ultrasonography of the eye.

The above data were all taken under conditions which optimized reflection and absorption path geometry. Such optimum conditions cannot be achieved under actual operating conditions. Geometric restrictions are imposed by the inherent shape of the structure and its relationship to adjacent structures, resulting in weaker echoes than those recorded in the data. These limitations can in part be overcome by rotating the eye and/or the transducer for the optimum geometric path and by modification of the transducer design.

Summary

The principles of operation of an ultrasonic locator are explained and the methods of displaying the acoustic reflections diagrammed. The physiologic and physical limitations of such a system are evaluated.

Data and the methods used in measuring the acoustic properties of the eye are presented.

The data contained in this paper are fundamental to the interpretation of echograms of the eye. They provide an indication of the limitations of the system as well as laying the

foundation for the evaluation of pathologic changes in the eye.

The coefficients of absorption of the ocular tissues have also been determined. On the basis of the combined absorption and reflection findings it is concluded that ultrasonic visualization of the eye at 15 mc. is feasible. However, apical orbital studies may require a lower frequency since the absorption by the tissues is inverse to the frequency. It may be possible to examine the apex of the orbit by rotating the eye into such a position that the lens is moved out of the path of the ultrasonic beam, thus eliminating approximately 50 db. of absorption.

The ultrasonic locator is a new tool for examining the tissues of the eye without in any way modifying them or altering their physiologic state. The thickness of structures and the depths of the various chambers may be measured without the use of light. As a clinical instrument many heretofore inaccessible areas such as the interior of the light opaque eye and the orbit will be opened to exploration without the use of surgery.

333 King Street.

References

1. Mundt, G. H., Jr., and Hughes, W. F., Jr.: Ultrasonics in ocular diagnosis. Am. J. Ophth., **41**:488 (Mar.) 1956.

2. Begui, Z. E.: Acoustic properties of the refractive media of the eye. J. Accoustical Soc. Am., **26**: 365-368 (May) 1954.

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Young, Robert L.

ANNUAL BUSINESS MEETING

The annual business meeting of the association was called to order, Dr. Michael J. Hogan, chairman, presiding.

Chairman Hogan: The first order of business is the report of the secretary-treasurer.

Secretary Lorand V. Johnson: The auditor's report has been approved by the auditing commmittee and is printed in the spring supplement section. I regret that distribution of the supplement could not be made before this meeting, but it will accompany the July issue of The American Journal of Ophthalmology.

The expenses for fiscal 1957 were $7,201. Cash on hand in the bank January 1, 1958, $10,451.19. New members voted into membership in May, 1958, were 225. The total paid membership as of May, 1958, is 1,056 members.

Chairman Hogan: The next order of business is the report of the nominating committee. Dr. Schlaegel, chairman, is not here. Other members of this committee are Drs. Frederick Blodi, Elmer Ballintine, and Theodore Sanders.

The nominating committee selected Dr. John Harris for trustee. I will entertain a motion from the floor that Dr. Harris be selected as the incoming trustee.

Dr. Blodi: I so move.

Dr. Peter C. Kronfeld (Chicago): I second the motion.

(The motion was put to a vote and was carried unanimously.)

Chairman Hogan: Dr. Lorand Johnson has been nominated as secreatry-treasurer. Are there other nominations?

Member: I move that Dr. Johnson be re-elected secretary-treasurer.

(The motion was severally seconded, was put to a vote, and was carried unanimously.)

Chairman Hogan: At the trustees' meeting it was voted that Dr. Georgiana Theobold be awarded an honorary membership in the association. May I have such a motion?

Member: I so move.

(The motion was severally seconded, was put to a vote, and was carried unanimously.)